

**Modulation of Host Death by *Chlamydia trachomatis* — the Role of the *Chlamydia*-specific Protease CPAF**

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## Abstract

*Chlamydia* is confirmed to modulate host cell death pathways to complete its own developmental cycle. A balance between pro- and anti- apoptotic influences by *Chlamydia* has been postulated.

In this study, the effect of *Chlamydia trachomatis* on activation of host cell death pathways was investigated. *C. trachomatis* infection induced caspase-3-independent cell death, without the stimulation of apoptotic factors like cytochrome *c* and apoptotic induction factor (AIF). On the other hand, after treatment with staurosporine, the activated caspase-3 and the subsequent apoptotic nuclear fragments displayed in uninfected cells were inhibited by chlamydial infection.

Poly (ADP-ribose) polymerase-1 (PARP-1) is known to play an important role on regulating apoptosis and necrosis. During necrosis, it will be over-activated and degraded differently from that observed during apoptosis, which exhibited the signature of 89 kDa and 24 kDa fragments. After *Chlamydia* infection, however, PARP-1 was cleaved to necrotic-like multi-fragment independent of caspase-3 activation. Strikingly, this cleavage was accompanied by a highly decreased enzymatic activity. PARP-1 silencing by siRNA in the host cells resulted in cell death similar to that induced by *Chlamydia* infection, but has no effect on chlamydial replication.

Chlamydial but not host cell protein synthesis contributed to this PARP-1 cleavage. Cell free degradation assay confirmed that this proteolytic activity only existed in cytosolic extractions of infected cells. The purified proteolytic protein fraction after column chromatography exhibited a 29 kDa fragment by Coomassie staining. 2D gel electrophoresis combined with mass spectrometry proved that this 29 kDa fragment corresponds to the NH<sub>2</sub>-terminal portion of chlamydial proteolytic activity factor (CPAF).

The high mobility group box-1 (HMGB-1) protein, which is known to be released to the extracellular matrix to induce inflammation during necrotic cell death, was also degraded by CPAF in the late stage of the chlamydial infectious cycle. This gives the suggestion that *Chlamydia* may evade the host immune system by degrading this inflammatory factor.

Under the stressful conditions like exposure to IFN- $\gamma$ , *C. trachomatis* underwent a special form so called “persistence”, which is characterized by enlarged pleomorphic RBs and reduced inclusion size, with minimal cultivability and low infectivity. The persistent

*Chlamydia* also exhibited the ability of apoptosis inhibition which ensures the long-term persistence in host cells.

During persistent chlamydial infection, the nuclear proteins PARP-1 and HMGB-1 were not degraded, whereas cell free degradation assays with the cytosolic extraction from persistently infected cells showed the activity to cleave both proteins, suggesting the CPAF translocation to the host nucleus was inhibited during persistence. The pro-apoptotic BH-3 proteins, degraded by CPAF during active infection, were only slightly degraded during persistence. RT-PCR gave the evidence that the expression of CPAF was highly decreased during persistent infection.

Our results gave the convincing evidence that the *Chlamydia*-secreted protease CPAF, as a ubiquitous protease in *Chlamydia*-infected cells, plays an important role in the regulation of host cell death pathways and fulfilling the requirements for chlamydial developmental cycle. During active infection this protease degrades many host factors, on the one hand, cleavage of nuclear proteins could induce the host cell instability and subsequent cell death, on the other hand, the degradation of pro-apoptotic BH-3 protein protect the infected cells against apoptotic stimuli. For the long-term persistence in host cells, *Chlamydia* highly decreases the expression of CPAF, which only existed in the cytosol but is not translocated to the nucleus. The nuclear proteins kept intact which may make sure the stability of the host cells, whereas slight degradation of pro-apoptotic BH-3 protein still support the persistent infection resistance to apoptosis.



## Zusammenfassung

Chlamydien sind obligat intrazelluläre Bakterien, die die Zelltod-Signalwege ihrer Wirtszellen modulieren, um ihren eigenen Entwicklungszyklus vollenden zu können. Es wurde ein Gleichgewicht zwischen zelltodinduzierenden und –inhibierenden Einflüssen von Chlamydien postuliert.

In dieser Studie wurde der Effekt von *Chlamydia trachomatis* auf die Aktivierung der Zelltod-Signalwege der Wirtszelle untersucht. Die Infektion von HeLa-Zellen mit *C. trachomatis* induzierte einen Caspase-3-unabhängigen Zelltod ohne Stimulierung verschiedener apoptotischer Faktoren, wie Cytochrom c und Apoptose-Induktions-Faktor (AIF). Auf der anderen Seite inhibierte die chlamydiale Infektion die durch Staurosporin verursachte Caspase-3-Aktivierung und die anschließende Bildung apoptotischer nukleärer Fragmente.

Poly (ADP-ribose) Polymerase-1 (PARP-1) spielt eine wichtige Rolle bei der Regulation von Apoptose und Nekrose. Während eines nekrotischen Zelltodes ist sie überaktiviert und zeigt nachfolgend ein anderes Degradierungsmuster im Vergleich zur Apoptose, welche durch die Spaltung von PARP-1 in 89 und 24 kDa-Fragmente gekennzeichnet ist. Nach Infektion mit Chlamydien hingegen wurde PARP-1 in mehrere Fragmente gespalten und zeigte ein Caspase-3-unabhängiges Muster. Auffallend war, dass diese Spaltung mit einer starken Reduktion der enzymatischen Aktivität einherging. Die Inhibition der PARP-1-Synthese in HeLa-Zellen mit Hilfe von siRNA induzierte einen Zelltod. Diese Ergebnisse weisen auf eine Rolle der PARP-1-Degradation bei der Induktion eines Zelltodes durch Chlamydien hin.

Die Spaltung von PARP-1 ist abhängig von der chlamydialen Proteinsynthese, jedoch nicht von der Wirtszellproteinsynthese. Proteolytische Assays im zellfreien System bestätigten, dass diese Aktivität nur in cytosolischen Extrakten infizierter Zellen existierte. Durch Säulenchromatographie aufgereinigte proteolytische Fraktionen von cytosolischen Extrakten zeigten nach Coomassie-Färbung ein 29 kDa-Fragment. Die Analyse mittels 2D-Gelelektrophorese und Massenspektrometrie bewiesen, dass dieses 29 kDa-Fragment dem NH<sub>2</sub>-terminalen Teil des chlamydial proteolytic activity factor (CPAF) entspricht.

Das high mobility group box-1 (HMGB-1) Protein, welches während eines nekrotischen Zelltods in die extrazelluläre Matrix abgegeben wird und als proinflammatorischer Faktor wirkt, wurde während der späten Phase des chlamydialen Infektionszyklus ebenfalls durch

CPAF degradiert. Durch die HMGB-1-Degradation könnten Chlamydien der Entzündungsreaktion und Abwehrmechanismen des Immunsystems entgehen.

Unter Stressbedingungen, z. B. der Stimulation von infizierten Zellen durch IFN- $\gamma$ , können Chlamydien intrazellulär persistierende Formen entwickeln. Diese sind durch vergrößerte, pleomorphe Retikularkörperchen charakterisiert, welche sich kaum noch replizieren und sich nicht in infektiöse Formen umwandeln. Diese Chlamydienformen zeigten ebenfalls die Fähigkeit zur Inhibition der Apoptose, welche offensichtlich die Langzeit-Persistenz in den Wirtszellen gewährleistet.

Während der persistenten chlamydialen Infektion wurden die nukleären Proteine PARP-1 und HMGB-1 nicht degradiert. Zellfreie proteolytische Assays zeigten hingegen, dass der cytosolische Extrakt persistent infizierter Zellen beide Proteine spalten konnte. Dies deutet darauf hin, dass die Translokation von CPAF in den Zellkern der Wirtszelle während der Persistenz unterdrückt wird. Die proapoptotischen BH3-Proteine, welche während der replikativen Infektion durch CPAF abgebaut wurden, waren während der Persistenz nur geringfügig degradiert. Mittels RT-PCR konnte bewiesen werden, dass die Expression von CPAF während der Persistenz stark reduziert war.

Die hier dargestellten Ergebnisse zeigen, dass die durch Chlamydien sezernierte Protease CPAF eine wichtige Rolle bei der Regulation von Zelltod-Signalwegen der Wirtszelle und bei der Komplettierung des chlamydialen Entwicklungszyklus spielt. Während der aktiven Infektion spaltet diese Protease eine Reihe von Wirtszellproteinen. Einerseits kann die Spaltung von Kernproteinen zur Instabilität der Wirtszelle und zum anschließenden Zelltod führen. Andererseits schützt die Degradation von proapoptotischen BH3-Proteinen die infizierten Zellen gegenüber Apoptose induzierenden Stimuli. Für die Langzeit-Persistenz in ihren Wirtszellen reduzieren Chlamydien die Expression von CPAF, welches unter diesen Bedingungen nur in geringen Mengen im Cytosol vorkommt und nicht in den Zellkern transloziert wird. Die geringgradige Degradation proapoptotischer BH3-Proteine im Cytosol kann die Apoptose-Resistenz der infizierten Zelle aber auch bei einer persistenten Infektion aufrechterhalten.

## Abbreviations

AIF	Apoptosis-inducing factors
BCIP/NBT	5-bromo-4-chloro-3-indolylphosphate tetrazolium chloride
BGM	Buffalo Green Monkey
BSA	Bovine serum albumin
CADD	<i>Chlamydia</i> protein associated with death domain
cDNA	Complementary deoxyribonucleic acid
CE	Cytosolic extracts
cHSP60	Chlamydial 60 kDa heat shock protein
cIAP	Inhibitor of apoptosis protein
CPAF	Chlamydial protease-like factor
DAPI	4'-6-Diamidino-2-phenylindole
DBD	DNA-binding domain
DISC	death inducing signalling complex
DNA	Deoxyribonucleic acid
EB	Elementary body
ELISA	Enzyme-linked immunosorbent assay
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPIC	Guinea pig inclusion conjunctivitis
HMGB-1	High mobility group box 1-protein
IAP	Inhibitor of apoptosis
IDO	Indoleamine 2, 3-dioxygenase
IFN- $\gamma$	Interferon gamma
IFU	Inclusion-forming units
IgG	Immunoglobulin G
LGV	Lymphogranuloma venereum

m.o.i.	Multiplicity of infection
MOMP	Major outer membrane protein
mRNA	Message ribonucleic acid
NE	Nuclear extracts
NF $\kappa$ B	Nuclear factor kappa B
PARP-1	Poly (ADP-ribose) polymerase-1
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
p.i.	Post infection
PI	Propidium iodide
PI3K	Phosphoinositide-3 kinase
PKC $\delta$	Protein kinase C $\delta$
PMF	Peptide mass fingerprint
RB	Reticulate body
RLU	Relative light units
RT-PCR	Reverse transcriptase polymerase chain reaction
siRNA	Small interfering RNA
TBS	Tris-buffered saline
TNF $\alpha$	Tumor necrotic factor $\alpha$
TNFR	Tumor necrosis factor receptor
TTSS	Type III secretion system

# 1 Introduction

## 1.1 Chlamydiae

Chlamydiae are gram-negative obligate intracellular bacteria which target epithelial cells of mucosae and replicate within specialized parasitophorous vacuoles. Different species of *Chlamydia* infect a variety of hosts with a wide range of tissue tropisms and varied diseases.

### 1.1.1 Taxonomy and pathogenesis

Chlamydiae have been placed in their own order, *Chlamydiales*, with the family of *Chlamydiaceae*, which was divided in two genera, *Chlamydia* and *Chlamydophila*, containing altogether nine species (Fig. 1. 1). The genus *Chlamydia* consists of three species, namely *C. trachomatis*, *C. suis* and *C. muridarum*, whereas *Cp. psittaci*, *Cp. abortus*, *Cp. felis*, *Cp. caviae*, *Cp. pecorum* and *Cp. pneumoniae* were placed under the genus *Chlamydophila*. Two of these species, *C. trachomatis* and *Cp. pneumoniae*, are common pathogens in humans, whereas the other species usually occur in animals.

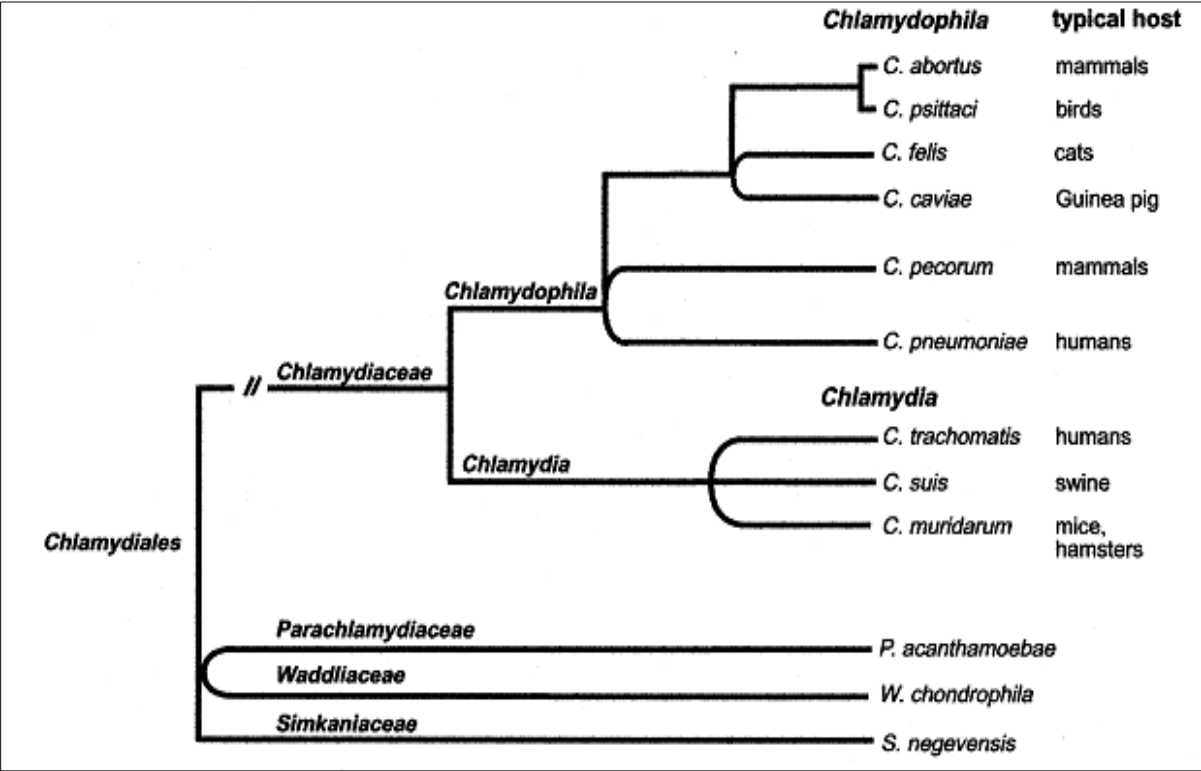


Fig. 1. 1 The classification of *Chlamydia* (Everett, K. D., 2001)

*C. trachomatis* causes severe infections of the epithelial tissue of eyes and urogenital tract. It is divided into three groups of serovars. Serovar A to C is the leading cause of preventable blindness in developing countries. Serovar D to K is responsible for the majority of sexually transmitted infections; ascending infection by these serovars of the female upper genital tract leads to ectopic pregnancy and tubal infertility (Carlin, J. M., 1995). These infections are also associated with reactive arthritis, which develops in 1 to 3% of patients after genital chlamydial infection (Wollenhaupt, J., 1990). Serovar L1 to L3 shares the unique ability to pass through epithelia, after which they disseminate, invade, and destroy lymphatic tissue and cause Lymphogranuloma Venereum (LGV) (Mabey, D., 2002).

*Cp. pneumoniae* is a respiratory pathogen that causes acute and chronic respiratory diseases. It is estimated to cause an average of 10% of community-acquired pneumonia cases and 5% of bronchitis and sinusitis cases (Kuo, C. C., 1995). Unresolved respiratory *Cp. pneumoniae* infection may contribute to the pathogenesis of chronic inflammatory lung diseases (Hahn, D. L., 1991; Blasi, F., 1993). In addition, it also acts as a possible risk factor for the development of atherosclerosis (Belland, R. J., 2004) or promoting the destabilization of atherosclerosis plaque (Rödel, J., 2004).

### 1.1.2 Developmental cycle

All chlamydial species share a common biphasic developmental cycle (Fig. 1. 2), which involves two distinct morphological and functional forms: the smaller ( $\approx 0.3 \mu\text{m}$ ) extracellular, metabolically inactive infectious particles called elementary bodies (EBs) and the larger ( $0.8\sim 1.0 \mu\text{m}$ ) intracellular metabolically active particles termed reticulate bodies (RBs) (Abelrahman, Y. M., 2005).

The EB has a pear shape and possesses a rigid outer membrane with the DNA packed by histone-like proteins. The rigidity of EBs is based on cross-linking of three cysteine-rich proteins. The most important of these is the major outer membrane protein (MOMP), which represents up to 60% of the weight of the outer membrane. The RB possesses a fragile membrane lacking the extensive disulfide bonds with tendency to form pleomorphic outer envelope blebs.

Intracellular growth of chlamydia is initiated by the invasion of non-phagocytic epithelial cells by infectious, metabolically inactive EBs. The internalized EBs replicate within a membrane-bound vacuole termed “inclusion” and differentiate into RBs. This includes the

DNA replication and reduction of the disulfide bridges of the outer membrane. RBs multiply by binary fission. After 8 to 12 rounds of multiplication, RBs begin to convert into EBs, including packing of the DNA and synthesis of late outer membrane proteins that are disulfide bridged. At 48 to 72 h post infection (p.i.), depending primarily on the infecting species, EBs progeny are eventually released through host cell lysis or extrusion (Moulder, J. W., 1991; Wolf, K., 2000) (Fig. 1. 2).

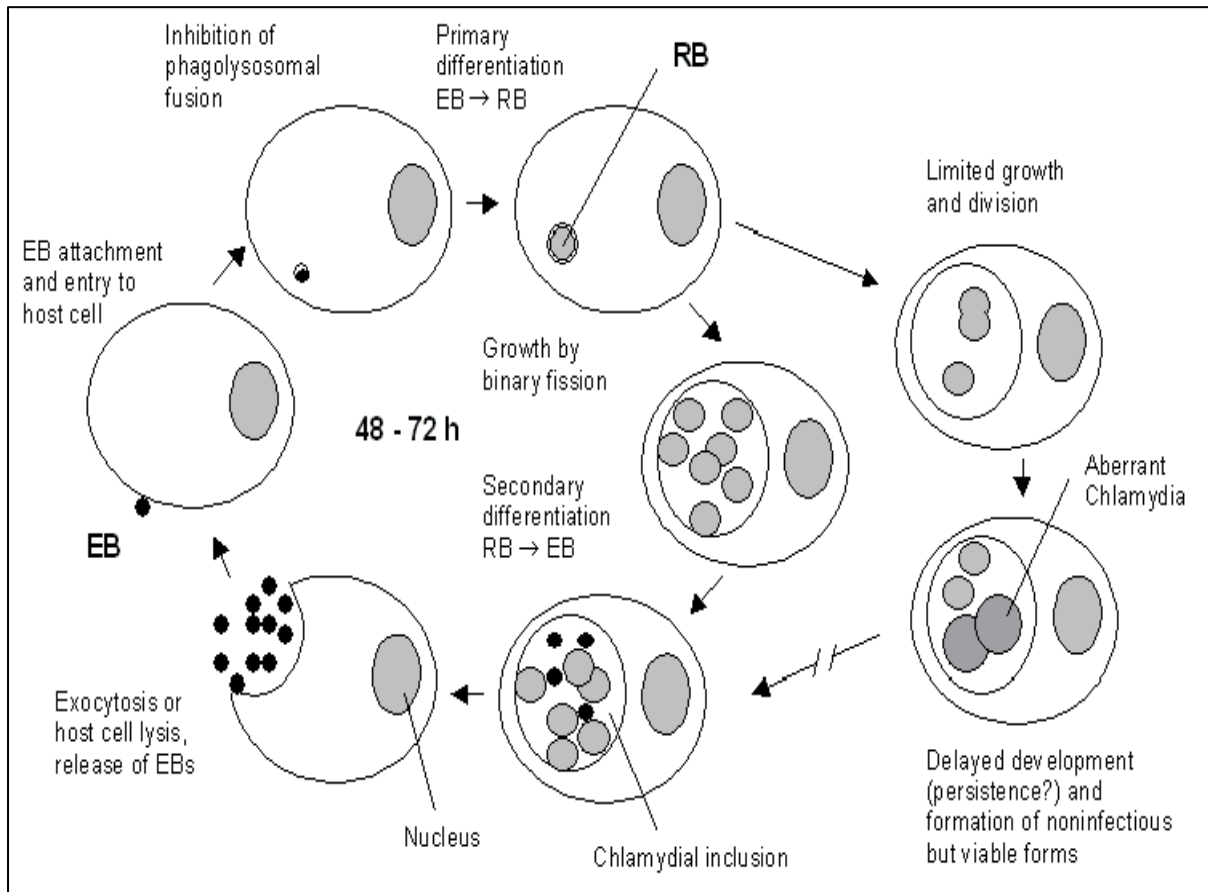


Fig. 1. 2 Developmental cycle of *Chlamydia*. (Beatty, W. L., 1994)

### 1.1.3 *Chlamydia* persistence

In response to stress conditions, chlamydiae can switch to a special persistent form, which is metabolically and morphologically altered and difficult to be eliminated by host cells (Hogan, R. J., 2004). Persistent chlamydia has been described as an atypical “aberrant” morphology, i.e., enlarged pleomorphic RBs and reduced inclusion size with minimal cultivability and low infectivity resulting in a long-term relationship with the infected host cell (Beatty, W. L., 1994) (Fig. 1. 2). The persistent chlamydial infection is thought to

correlate with many chronic infections, including pelvic inflammatory disease, scarring trachoma and reactive arthritis (Hogan, R. J., 2004).

*In vivo*, the host response against *Chlamydia* mostly depends on the inflammatory cytokine gamma interferon (IFN- $\gamma$ ). IFN- $\gamma$  suppresses *Chlamydia* mainly through the induction of tryptophan degrading enzyme indoleamine 2, 3-dioxygenase (IDO). IDO is responsible for tryptophan catabolism, thus decreasing the intracellular concentration of tryptophan available for chlamydia (Beatty, W. L., 1993). Chlamydia deprived of tryptophan fail to complete secondary differentiation into infectious EBs, resulting in the loss of infectivity and cell-to-cell transmission and undergoing to the persistent form.

However, different chlamydial species exhibit differential sensitivities to the effect of IFN- $\gamma$ . For example, the genital serovars (D to K as well as L1 to L3) but not ocular serovars (A to C and Ba) of the *C. trachomatis* displayed the ability to resist on IFN- $\gamma$  exposure. The differences in susceptibility of various chlamydial strains to IFN- $\gamma$  have been explained by the differences in their ability to acquire exogenous tryptophan or to synthesize their own tryptophan (Morrison, R. P., 2000; Caldwell, H. D., 2003).

Despite the evident importance of tryptophan catabolism, other mechanisms such as the inducible nitric oxide synthase effector pathway, iron deprivation and antibiotic treatment could also induce abnormal *Chlamydia* RBs. (Igietseme, J. U., 1998).

#### **1.1.4 Chlamydial effector proteins**

Once internalized, chlamydiae stay inside the inclusion throughout the intracellular stage, which lasts for 2 to 4 days depending on the chlamydial species. Like other gram-negative pathogens, chlamydiae translocate ‘effector’ proteins into their host cytosol to modulate cellular functions. Now it is confirmed that all *Chlamydiae* code for the core components of a type III secretion system (TTSS), a protein transport system used by gram-negative bacteria to translocate proteins into the cytoplasm of the host cell, suggesting *Chlamydia* may deliver effector proteins to the inclusion membrane and into the host cell cytoplasm by type III secretion (Peter, J., 2007; Clifton, D. R., 2004; Stephens, R. S., 1998; Hsia, R-c., 1997). So far, evidence for the secretion of chlamydial effector proteins by type III secretion has been limited to proteins associated with the inclusion membrane, such as CopN (Fields, K. A., 2000) and members of the family of inclusion membrane proteins (Inc) (Subtil, C. Parsot, 2001).



It should be noted that chlamydial effector proteins also access the cytoplasm of infected cells via TTS-independent mechanisms. For example, chlamydial protease-like activity factor (CPAF) contains a domain characteristic of bacterial Tail-specific proteases (Tsp) (Shaw, A. C., 2002) and is secreted to the inclusion lumen before translocation into the cytoplasm of the infected cells (Heuer, D., 2003).

Throughout the infectious cycle *Chlamydia* modulate many cellular functions, which require the activity of chlamydial effector proteins, including subversion of the cytoskeleton to facilitate intracellular redistribution of newly internalized EBs, inhibition of apoptosis to ensure intracellular survival during the developmental cycle, induction of cell death to release chlamydial progeny upon completion of the cycle, and evasion of host immune system recognition (Carabeo, R. A., 2002). For example, the Incs are probably central regulators of bacterial-host interactions. IncA mediates homotypic fusion of inclusions potentially by forming a SNARE-like fusogenic intermediate between adjacent inclusions (Hackstadt, T., 1999; Delevoye, C., 2004). IncG sequesters the host protein 14-3-3 $\beta$  and its pro-apoptotic binding partner phosphor-BAD (Verbeke, P., 2006).

Another prominent function of these effector proteins is to interfere with the host cell death programs which are central to innate immune responses. During middle stage of infection, the CPAF degrades pro-apoptotic BH-3 proteins to shut down the infected cell's ability to undergo apoptosis in response to intrinsic and extrinsic stimuli. Finally, *Chlamydia* infection significantly impacts the cell cycle of infected cells by cleavage of the mitotic cyclin B1 and delays in cytokinesis (Balsara, Z. R., 2006; Greene, W., 2003). These later functions lead to genomic instability, which in conjunction with the strong anti-apoptotic effect of chlamydial infection may explain how *Chlamydia* modulate host cell death programs to fulfil its unique intracellular parasite lifestyle.

## 1.2 Cell death

In general, cellular homeostasis is maintained by a balance between proliferation and cell death. Two major types of cell death are described as apoptosis and necrosis.

### 1.2.1 Apoptosis

Apoptosis is a well-conserved process that multicellular organisms use for control of development and homeostasis as well as the removal of unwanted cells recognized by the

immune system. Common morphological and biochemical features of apoptosis include nuclear chromatin condensation, cytoplasmic shrinking, dilated endoplasmic reticulum, blebbing of plasma membrane, phosphatidylserine asymmetry, internucleosomal DNA fragmentation, reduction of mitochondrial transmembrane potential, intracellular acidification, etc (Vaux, D. L., 1994; Wyllie, A. H., 1997; Golstein, P., 1991).

Apoptosis can be induced through both extrinsic and intrinsic pathways (Hengartner, M. O., 2000; Krammer, P. H., 2000; Green, D. R., 1998; Salvesen, G. S., 2002). The intrinsic pathway involves the activation of several pro-caspases followed by the mitochondria released apoptotic factors. The extrinsic pathway involves the activation of the death receptors, FAS and tumor necrosis factor receptors (TNFR). In most cases, both pathways require the release of mitochondrial cytochrome *c*, which in turn binds to Apaf-1 and caspase 9 to form an apoptosome, the caspase-activating signaling complex (Ekert, P. G., 2005). Subsequently, caspases get activated and cleave cystein-containing proteins like lamins, poly (ADP-ribose) polymerase 1 (PARP-1), histones, nucleolin, and fodrin, thus leading to cell death (Fig. 1. 3). Now, the cleavage of PARP-1 in fragments of 89 kDa and 24 kDa by activated caspase-3 and 7 has become a useful hallmark of apoptosis.

The release of cytochrome *c* is regulated by Bcl-2 family proteins. This family consists of three groups of proteins: the pro-apoptotic subfamily (Bax, Bak and Bok), which directly alters the integrity of the mitochondrial membrane and causes cytochrome *c* release; anti-apoptotic proteins Bcl-2 and Bcl-xl, which inhibit cytochrome *c* release; and the BH-3 only proteins that exhibit pro-apoptotic activity by inhibition of Bcl-2 or Bcl-xl. c-FLIP is a critical negative regulator of the extrinsic cell death pathway. It functions as a competitive inhibitor of pro-caspase 8. Upon death receptor stimulation, c-FLIP can be efficiently recruited to the death inducing signalling complex (DISC) and inhibit pro-caspase 8 activation at the DISC (Zhang, N., 2005).

Two types of cells have been identified with regards to the requirement for a mitochondrial amplification loop during apoptosis. In type II cells (HeLa, Jurkat, and human CEM T-cell line), apoptosis inducers can activate caspase-3 only through a mitochondrion-dependent step. In type I cells (human B lymphoblast cell line SKW6.4, H9, BJAB), caspase-8 recruitment and activation by cell surface receptors can lead directly to caspase-3 activation without help from mitochondria (Barnhart, B. C., 2003).

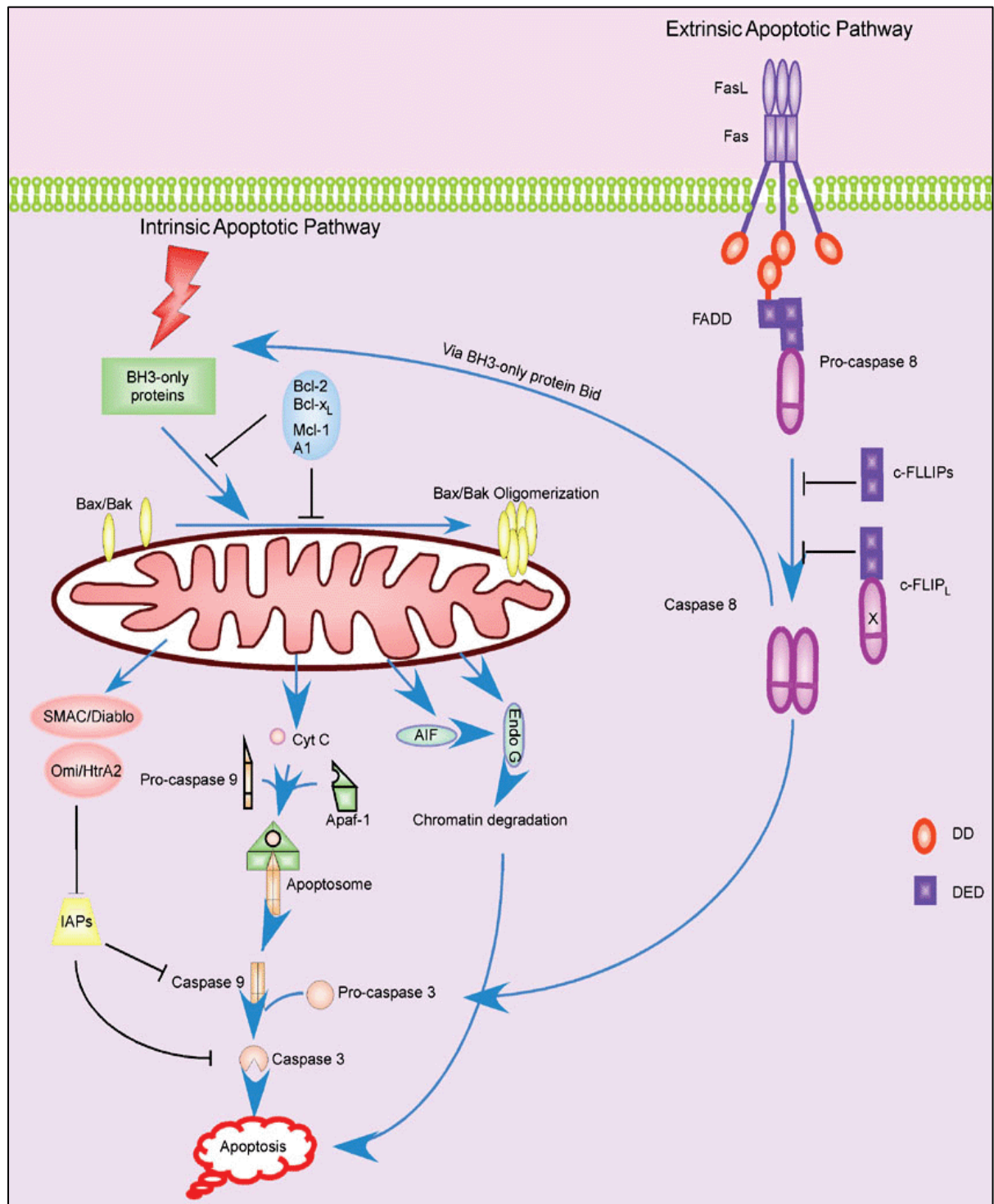


Fig. 1. 3 Apoptotic pathway (Zhang, N., 2005)

### 1.2.2 Necrosis

In contrast to apoptosis, necrosis has generally been considered as an unregulated form of cell death which is associated with acute injury to cells. It results in plasma membrane disintegration and leakage of cellular contents to stimulate inflammation (Cohen, G. M., 1997; Fiers, W., 1999).

Necrosis has long been described as a consequence of extreme physico-chemical stress, such as heat, osmotic shock, mechanical stress, freeze-thawing and high concentration of hydrogen peroxide. Under these conditions, cell death occurs quickly due to the direct effect of the stressor on the cell, and therefore this cell death process has been described as accidental and uncontrolled. However, recently many different cellular stimuli (TNF on certain cell lines, dsRNA, ATP depletion, ischemia) have been shown to induce a necrotic process that follows defined steps and signalling events reminiscent of a true cell death program (Vanden Berghe, T., 2007). This induced necrotic cell death results from extensive crosstalk between several biochemical and molecular events at different cellular levels, and it is similarly controlled like apoptosis.

It is important to distinguish necrosis from other forms of cell death, particularly because it is often associated with unwarranted loss of cells in human pathologies. It can also lead to local inflammation due to release intracellular factors from dead cells; the so-called damage associated molecular patterns that alert the innate immune system. Now the different cleavages of PARP-1 have become the method to distinguish apoptosis and necrosis. During necrosis, the PARP-1 is cleaved to the multi-fragment form (72, 55 and 42 kDa) by the lysosomal proteases (Gobeil, S., 2001). This necrotic PARP-1 cleavage could also induce the release of the high mobility group box 1 protein (HMGB-1), which further stimulates monocytes to secrete a subset of pro-inflammatory cytokines and act as an important inflammatory factor (Ditsworth, D., 2007; Müller, S., 2001).

However, there is no other clear biochemical definition of necrotic cell death and consequently no specific biochemical markers that unambiguously discriminate necrosis from apoptosis. Another problem is that even the interpretation of dying cell morphology may be complex, because in the absence of phagocytosis apoptotic cells proceed to a stage called secondary necrosis, which shares many features with primary necrosis.

### 1.3 *Chlamydia* regulates host cells death pathway

As an intracellular pathogen, *Chlamydia* replicates strictly in a vacuole in the host cytosol. Thus, it is essential to maintain the integrity of host cells during its developmental cycle not only for supplying the nutrients but also for protecting the intracellular organisms from host phagocytosis and antigen-specific immune effectors. However, at the later stage, the host cells rupture benefits on the release of elementary bodies to start a new infectious cycle. Therefore, modulation of the host cell death could be a relevant component of the chlamydial developmental cycle.

#### 1.3.1 *Chlamydia* inhibits apoptosis

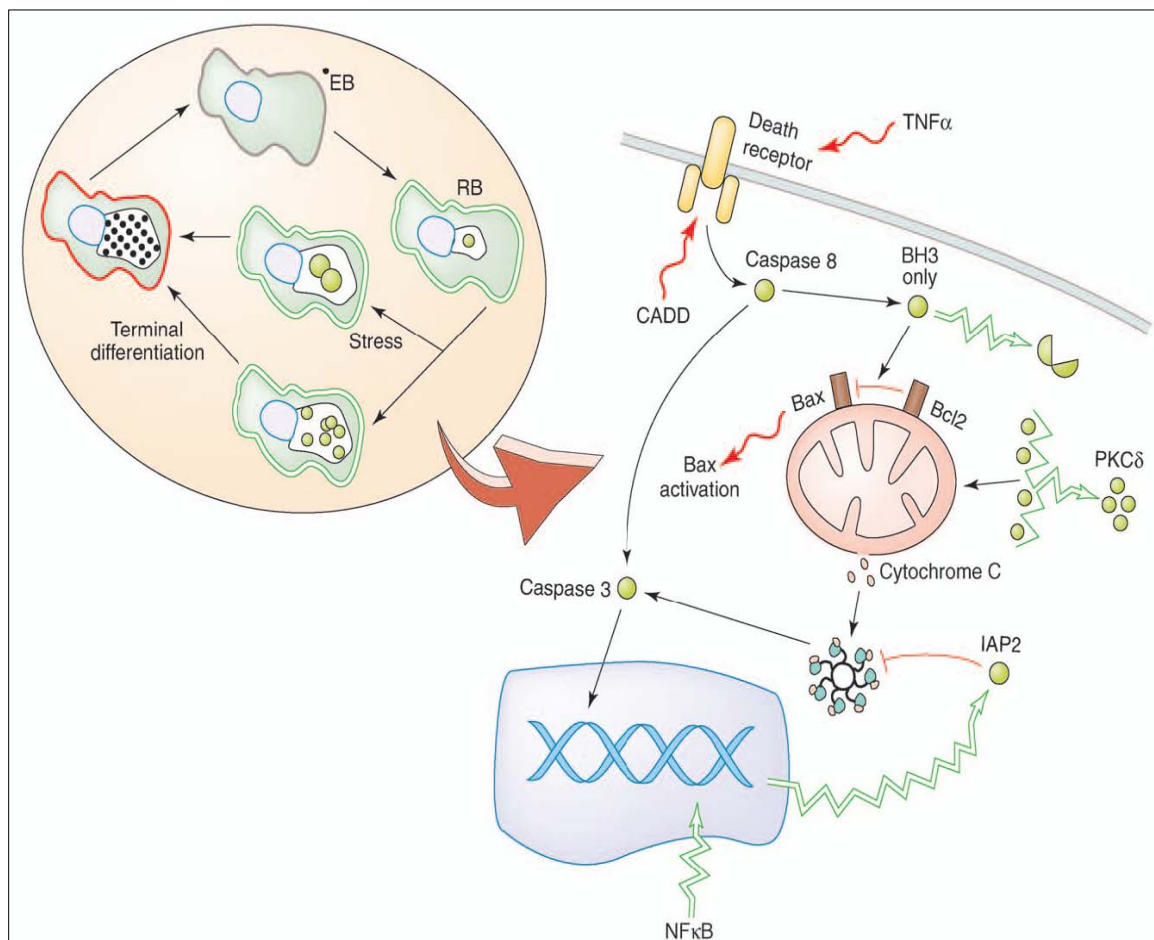
The anti-apoptotic activity of *Chlamydia* has consistently been demonstrated by its ability to inhibit chemically and spontaneously induced apoptosis during mid to late stages in the developmental cycle. The paper in 1998 first described that, *C. trachomatis*-infected cells are resistant to numerous experimental apoptotic stimuli, exhibiting inhibition of caspase activation and blockage of cytochrome *c* release from mitochondria (Fan, T., 1998). Later, several studies have shown that epithelial cells or monocytes that are infected with *C. trachomatis* or *Cp. pneumoniae* are protected against cell death stimuli (D. Dean, 2001; Fischer, S. F., 2001, 2004; Rajalingam, K., 2001; Greene, W., 2004).

An explanation for these observations could be the proteolytic cleavage of a wide range of pro-apoptotic BH-3 only proteins (Fischer, S. F., 2004). It is now known that *Chlamydia* are capable to degrade the pro-apoptotic proteins Puma, Bad, Noxa and tBid (Ying, S., 2005), a family of proteins that share this Bcl-2 homology domain. The secreted chlamydial protease CPAF was confirmed to be responsible for this degradation, thus ensuring a complete shut down of the infected cell's ability to undergo apoptosis in response to intrinsic and extrinsic stimuli.

Besides the BH-3 only proteins degradation, chlamydial infection also results in activation of the phosphoinositide-3 kinase (PI3K) pathway. During infection with *C. trachomatis*, activation of PI3K leads to Akt activation and in turn to phosphorylation of a pro-apoptotic protein, Bad. *C. trachomatis* prevents phosphorylated Bad from binding to mitochondria by sequestering their binding partners, 14-3-3 $\beta$  proteins, via a chlamydial inclusion membrane protein IncG (Verbeke, P., 2006).

Additionally, *Chlamydia* appears to be capable of accumulating diacylglycerol in the inclusion, which recruits and sequesters pro-apoptotic effector protein kinase C  $\delta$  (PKC $\delta$ ). When PKC $\delta$  is activated, it is cleaved to release a catalytically active fragment that translocates to the mitochondria where it promotes the release of cytochrome *c* and subsequently activates of the apoptotic pathway (Tse, S. M., 2005). Thus, chlamydial recruiting PKC $\delta$  could protect the mitochondria against the death signal.

Anti-apoptotic gene regulation through nuclear factor NF- $\kappa$ B-mediated gene activation is an anti-apoptotic mechanism used by other pathogens, but conflicting results exist for *Chlamydia*. *Cp. pneumoniae* infection of a human monocytic cell line induced expression of nuclear factor kappa B (NF- $\kappa$ B) and the inhibitor of apoptosis 2 (IAP2). Inhibition of NF- $\kappa$ B by proteasome inhibitor MG132 resulted in inhibition of IAP2 and induction of apoptosis (Wahl, C., 2003). However, other studies is failed to demonstrate the function of NF- $\kappa$ B activation in *C. trachomatis* inhibition of apoptosis. Neither chemical inhibition nor gene deletion of NF- $\kappa$ B had any effect on anti-apoptotic activity of *C. trachomatis* (Xiao, Y., 2005; Fischer. S. F., 2003).



**Fig. 1. 4** The inhibition of apoptosis by *Chlamydia* (Miyairi, I., 2006)

### 1.3.2 *Chlamydia* induces cell death

Cytotoxicity associated with chlamydial infection has been reported for many years. *Chlamydia* infection was thought to be lytic for the host cells. Productive cell culture infections ultimately result in host cell lysis (Abdelrahman, Y. M., 2005). Cell death seems to occur in uninfected neighboring cells or in infected cells at later stages of infection. Though the cells displayed some morphological characteristics of apoptotic death, there is no classical caspase activation (Ojcius, D. M., 1998; Schoier, J., 2001; Dumrese, C., 2005). Another publication suggested an additional possibility, namely, that *Cp. pneumoniae* induces a form of cell death which displays some apoptotic features, but is more consistent with necrosis. The term “aponecrosis” has been proposed to characterize this special death form induced by *Chlamydia* infection (Dumrese, C., 2005). These findings suggest that an ongoing death stimulus by chlamydial infection exists to induce the host cell death in the late stage of infection.

It is indicated that the pro-apoptotic protein Bax is activated during *Chlamydia* infection with the guinea pig inclusion conjunctivitis (GPIC) serovar of *Cp. psittaci* (J. L. Perfettini, 2002), giving an explanation for the *Chlamydia* induced caspase-independent cell death. One *Chlamydia* protein associated with death domain (CADD)— an iron-containing redox enzyme unique to chlamydial species, can induce cell death when ectopically expressed in mammalian cells by interacting with death domains of tumor necrosis factor (TNF) family of receptors (Stenner-Liewen, F., 2002). Host cytokines such as tumor necrotic factor  $\alpha$  (TNF- $\alpha$ ) (Jendro, M. C., 2004) have been implicated as sources of pro-apoptotic stimuli. *Cp. pneumoniae* infection is also known to modulate oxLDL-induced endothelial cell death, decreasing apoptosis and promoting necrosis (Nazzal, D., 2006). In addition, *Chlamydia*-induced necrosis might be associated with a heterogeneous family of clostridia cytotoxin homologues that are coded by the genomes of *C. trachomatis* and *Cp. psittaci* (Belland, R. J., 2001).

### 1.3.3 Chlamydial persistence regulate cell death

During persistent infection, the antigenic profile of *Chlamydia* differs from that of acute infection. Beatty *et al.* (1993, 1994) proposed that during chlamydial persistence, the reduced levels of MOMP, an immunoprotective antigen, could enable chlamydiae to avoid the development of protective immunity. On the other hand, steady of chlamydial 60 kDa heat shock protein (cHSP60) levels would promote immunopathology of persistent

chlamydia. Hypersensitivity reactions are known to be elicited by cHSP60 (Morrison, R. P., 1989, 1991; Patton, D. L., 1994), which is confirmed to induce apoptosis (Equils, O., 2006); suggesting that an apoptotic block may favour a chlamydial form that expresses inflammation-inducing proteins.

It has been reported that cells infected with *C. trachomatis* in the presence of IFN- $\gamma$  resist apoptosis due to external ligands, via inhibition of caspase activation (Dean, D., 2001). During persistent chlamydial infection, cytochrome c remained sequestered in the mitochondrial fraction after apoptotic induction (Deborah, D., 2001). The mechanism of protection in persistently infected cells seems to be the same as that observed in the absence of IFN- $\gamma$ .

Furthermore, anti-apoptotic activity is prolonged during chlamydial persistence, which strengthens the hypothesis that active chlamydial metabolism maintains host cell integrity and contributes to intracellular survival (Dean, D., 2001; Perfettini, J. L., 2002). However, the precise mechanisms and factors that *Chlamydia* uses to modulate host cell death pathways during different life styles still need to be further investigated.



## 1.4 Aim of this work

In summary, to fulfill the long-term intracellular survive, *Chlamydia* is confirmed to inhibit the death stimuli for the host cells during the developmental cycle and spontaneously induce some kind of cell death to release the reproduced EBs. When this pathogen is under the conditional stress like exposure to IFN- $\gamma$ , it goes to a persistent form, which also exhibits abilities to protect the host cells against death stimuli and prevent the elimination by the host immune system. However, until now, the precise factors and mechanisms of how *Chlamydia* regulates host cell death pathways to fulfill its specific developmental cycle and the differences on this regulation between active and persistent infection are still unclear. It is unclear how the balance between cell death induction versus apoptosis inhibition is regulated in *Chlamydia* infected cells and which factors are involved.

The objective of my work can be described as follows:

1. To characterize the host cell death induced by *C. trachomatis* and to illuminate the mechanisms which *Chlamydia* used to regulate host death pathways.
2. To detect the factors like nuclear proteins PARP-1 and HMGB-1, which are involved in keeping host cell integrity and homeostasis, and to investigate the function of these factors in *Chlamydia* induced host cell death.
3. To compare the differences of cell death regulation between active and persistent infection, and explain how *Chlamydia* can modify host cell death pathways under different conditions.

## 2 Material and methods

### 2.1 Material

Strain	Company
<i>C. trachomatis</i> serovar D strain IC Cal 8	Institute of Ophthalmology, London, United Kingdom

Cell lines and culture medium	Company
BGM cell	American Type Culture Collection, ATCC; Manassas, VA
Fetal calf serum	PromoCell, Heidelberg, Germany
HeLa cell	ATCC; Manassas, VA
Minimal Essential Medium (OptiMEM, Gibco)	Invitrogen, Karlsruhe, Germany
Panserin medium	Panbiotech
SF-3 medium	Cytogen, Lohmar, Germany

Material	Company
25-cm <sup>2</sup> flasks	CellStar; Greiner Bio-One GmbH, Frickenhausen, Germany
6-well plates	Nunclon™, Roskilde, Denmark
8-well LabTec chamber	Nunclon™, Roskilde, Denmark
96-well plates	Nunclon™, Roskilde, Denmark
AKTA purifier 10 instruments	Pharmacia Biotech Inc
IPG dry strips	GE Healthcare Life Sciences
Mono Q ion exchange column	Amersham Pharmacia Biotech Inc.
Semidry transblot system	Bio-Rad, Munich, Germany

<b>Buffer and solution</b>	<b>Compound</b>
Bjerrum and Schafer-Nielsen Transfer buffer	48mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol (pH ~9)
Blocking buffer	4% bovine serum albumin, 0.05% Tween, TBS
Buffer B for Cell free degradation	0.5 mM NaCl, 1% Triton X-100 in 20 mM Tris (pH 8.0)
Buffer A for chromatography	0.01 M Tris, pH 7.2
Buffer B for chromatography	0.01 M Tris, 1 M NaCl, pH 7.2)
Equilibration buffer	6 M urea, 30% (w/v) glycerol and 2% (w/v) SDS in 0.05 M Tris-HCl buffer, pH 8.8.
Gel fixing solution	40% methanol, 7% glacial acetic acid
Gel staining solution	10% methanol, 0.1% CBB G-250, 1.6% <i>o</i> -phosphoric acid, and 10% (w/v) ammonium sulfate
HiPerfect transfection reagent	Qiagen, Hilden, Germany
IPG blocking buffer	7 M urea, 2 M thiourea, 2% CHAPS, 0.002% bromophenol blue, 0.5% IPG buffer, 1.2% De-Streak Reagent
Laemmli sample buffer	Bio-Rad, Munich, Germany
Neutralised solution	0.1 M Tris-base titrated to pH 6.5 with <i>o</i> -phosphoric acid
Saccharose phosphate buffer	PBS with 0.2 M saccharose and 2% FCS
<b>Chemical</b>	<b>Company</b>
Aprotinin	Sigma, US
Bovine serum albumin (BSA)	Sigma, US
Chloroamphenicol	Biocat, Heidelberg, Germany
Cycloheximide	Biocat, Heidelberg, Germany
DAPI	Invitrogen
DMSO	Sigma, US

Gel strengthener	Molecular Probes, Leiden, The Netherlands
Hoechst 34580	Invitrogen, Karlsruhe, Germany
Lactacystin	Enzo Life Sciences, Lörrach, Germany
Leupeptin	Serva, Heidelberg, Germany
Methanol	Mallinckrodt Baker, Deventer, Holland
Paraformaldehyde	Sigma, US
Phenylmethylsulfonyl fluoride (PMSF)	Serva, Heidelberg, Germany
Propidium iodide (PI)	Biotium, purchased from Biotrend, Cologne, Germany
Protein standard Mark 12	Invitrogen, Karlsruhe, Germany
SDS-PAGE Standards Broad Range	Bio-Rad, Munich, Germany
Sodium dodecyl sulfate (SDS)	Sigma, US
Staurosporine	Roche Applied Science, Mannheim, Germany
Sulforhodamine 101-annexin V	Biotium, Hayward, CA
SYBR Green I	(Molecular Probes; MoBiTec; Göttingen, Germany)
TBS-tween	Sigma, US
Trypsin	Biochrom AG

**Kits****Company**

Annexin V-FLUOS staining kit	Roche Applied Science, Mannheim, Germany
Caspase-Glo <sup>®</sup> 3/7 Assay	Promega, Mannheim, Germany
DEVD-NucView <sup>™</sup> caspase-3	Biotium, Hayward, CA
HMGB-1 enzyme-linked immunosorbent assay	Shino-test, Kanagawa, Japan
HP validated small interfering RNA (siRNA)	Invitrogen, Carlsbad, CA

Promega reverse transcription system	Promega, Mannheim, Germany
Rnase-Free Dnase Set	Qiagen, Hilden, Germany
RNeasy Mini Kit	Qiagen, Hilden, Germany
Universal colorimetric PARP-1 assay kit	R&D Systems, Wiesbaden-Nordenstadt, Germany

Antibody	Company
5-bromo-4-chloro-3-indolylphosphate toluidine salt- <i>p</i> -nitroblue tetrazolium chloride (BCIP/NBT substrate)	Sigma Fast, Sigma-Aldrich
Alkaline phosphatase-conjugated goat anti- rabbit/mouse IgG	Dianova, Hamburg, Germany
Mouse monoclonal antibody to chlamydial CPAF	Kindly provided by Dr. V. Forsbach-Birk, Institute of Medical Microbiology, University of Ulm, Germany
Mouse monoclonal antibody to <i>Chlamydia</i> <i>trachomatis</i> heat shock protein 60 (cHSP60)	Acris, Hiddenhausen, Germany
Mouse monoclonal antibody to <i>Chlamydia</i> <i>trachomatis</i> (MOMP)	Acris
Rabbit polyclonal antibody to human Bad	Cell-Signalling Tech
Rabbit polyclonal antibody to human Bim (BOD)	Sigma, US
Rabbit polyclonal antibodies to GAPDH	Santa Cruz Biotechnology
Rabbit polyclonal antibody to human HMGB-1	Abcam, Cambridge, UK
Rabbit polyclonal antibody to IDO	Santa Cruz Biotechnology
Rabbit polyclonal antibody to human Puma/bbc3, N-terminal	Sigma, US
Texas-conjugated goat anti-rabbit/ mouse IgG (H+L)	Santa Cruz Biotechnology

Primer	Sequence
16S rRNA of <i>Mycoplasma</i>	5'-ccagactcctacgggaggca-3' 5'-tgcgagcatactactcaggc-3'
cIAP-1	5'-cacatgcagctcgaatgagaa-3' 5'-tccaaggcagatttaaccacagg-3'
cIAP-2	5'-catgcagcccgcgtttaaatttc-3' 5'-cattccacggcagcattaatcac-3'
HP validated small interfering RNA (siRNA) targeting PARP-1 (SI02662989)	Qiagen, Hilden, Germany
nonsilencing All Stars Negative Control siRNA	Qiagen, Hilden, Germany
PDH	5'-ggtatggatgaggacctgga-3' 5'-cttccacagccctcgactaa-3'

Machine	Company
Branson Sonifier W-250	Heinemann, Germany
Confocal laser scanning microscope (TCS SP5)	Leica, Heidelberg, Germany
Confocal laser scanning microscope (Zeiss)	Zeiss, Jena, Germany
Ettan DALT System	GE Healthcare Life Sciences
FACSCalibur flow cytometer	BD Biosciences, Heidelberg, Germany
Fluorescence microscope	Axioskop, Zeiss, Jena, Germany
IPGphor <sup>TM</sup>	GE Healthcare Life Sciences
Semidry transblot system	Bio-Rad, Munich, Germany
Protean II xi multicell system	Bio-Rad, Munich, Germany
TRIO-Thermoblock	Biometra, Göttingen, Germany
Ultraflex I device	Bruker Daltonics, Bremen, Germany

Software	Company
Cell QuestPro	BD Biosciences, Heidelberg, Germany
Labscan software 5	GE Healthcare Life Sciences
MASCOT interface (MASCOT 2.1.0)	Matrix Science, London, UK
ProFound	Genomic Solutions, USA

## 2.2 Methods

### 2.2.1 Cell culture and *Chlamydia* organisms

HeLa cells (ATCC CCL-2) were grown in minimal essential medium with 10% fetal calf serum (FCS).

*C. trachomatis* serovar D strain IC Cal 8 (obtained from the Institute of Ophthalmology, London, United Kingdom) was propagated in buffalo green monkey (BGM) cells. *C. trachomatis* D were inoculated onto cell monolayers in 25-cm<sup>2</sup> flasks and centrifuged at  $2,000 \times g$  for 45 min at 37°C. The inoculum was removed and replaced with serum-free SF-3 medium. Infected cells were collected in phosphate-buffered saline (PBS) with 0.2 M sucrose and 2% FCS 48 h after infection and lysed by sonication. The chlamydial suspension was centrifuged at  $800 \times g$  to remove cellular debris. Supernatants were stored at -70°C. Infectivity titers of chlamydial stocks were quantified by titrating the number of inclusion-forming units (IFU) per milliliter in BGM cells. Mycoplasma contaminations in cell cultures were excluded by PCR targeting the 16S rRNA gene of *Mycoplasma*, *Acholeplasma*, and *Ureaplasma* species (Hotzel, H., 1998).

### 2.2.2 Infection and induction of persistence

For infection experiments, HeLa cells were grown in 35-mm-diameter culture wells (six-well plates) or 11-mm-diameter culture tube containing a glass coverslip (shell-vials) to c. 70% confluence. The cells were inoculated with *C. trachomatis* at a multiplicity of infection (m.o.i.) of 5. For mock-infected cultures diluted harvests of uninfected BGM cells were added. After centrifugation at  $4,000 \times g$  at 37°C for 45 min, the inoculum was decanted, and the cells were further incubated with OptiMEM containing 10% FCS.

To induce the persistence, HeLa cells were preincubated 48 h before infection with different concentration of IFN- $\gamma$ . After infection, the medium were changed and new IFN- $\gamma$  was added.

For inhibition experiments, the prokaryotic protein synthesis inhibitor chloroamphenicol (60  $\mu\text{g}/\text{mL}$ ) or the eukaryotic protein synthesis inhibitor cycloheximide (10  $\mu\text{g}/\text{mL}$ ) were added to the cultures directly after infection.

### **2.2.3 Immunofluorescence staining of *Chlamydia trachomatis***

Infected HeLa cell monolayers grown on coverslips were fixed with methanol at 48 h post infection. Samples were incubated with a FITC-conjugated antibody to the *C. trachomatis* major outer membrane protein (MOMP).

The number of inclusions per shell vial was calculated from determination of inclusions in 20 randomly selected  $\times 400$  microscopic fields under a fluorescence microscope.

### **2.2.4 Detection of cell death by PI/Hoechst staining and flow cytometry**

HeLa cells grown and infected in 8-well LabTec chambers were washed with PBS at 48 h after infection and fixed with 4% paraformaldehyde for 30 min. Then the cells were incubated with 10  $\mu\text{M}$  of Hoechst 34580 to stain DNA and propidium iodide (PI) to stain dead cells. Images were taken with a confocal laser scanning microscope.

For the identification of cell death by flow cytometry, the Annexin V-FLUOS Staining Kit was used to detect Phosphatidylserine exposure and PI incorporation. Culture supernatants were collected and mixed with trypsinized cells. After centrifugation at  $3000 \times g$  for 4 min, samples were washed in PBS and resuspended in the supplied buffer containing annexin V-FLUOS labeling reagent and PI. After 20 min incubation at room temperature, labeled cells were analyzed with a FACSCalibur flow cytometer and Cell QuestPro Software. Then thousand cells were scored for each sample.

### **2.2.5 Apoptosis induction and assay for nuclear morphology**

To induce apoptosis, cells with or without chlamydial infection were treated with 1  $\mu\text{M}$  staurosporine for 4 h. For nuclear morphology assays cells grown on coverslips were used for infection. After apoptosis induction cells were washed twice with PBS, fixed with methanol for 5 min at room temperature, and stained with 1  $\mu\text{M}$  DAPI at 37  $^{\circ}\text{C}$  for 30 min.



After being washed three times with PBS, nuclear fragmentation was examined under a fluorescence microscope. Nuclei in 10 randomly selected visual fields were counted and the percentage of apoptotic cells was calculated.

### **2.2.6 Localization of active caspase-3, cytochrome *c*, and AIF**

For the detection of active caspase-3 HeLa cells were cultured in 8-well LabTek chambers. Mock-infected and infected cells with or without staurosporine treatment were incubated with a caspase-3-specific chromogenic substrate (NucView 488 Caspase-3 Assay Kit), FITC conjugated-annexin V, and Hoechst 34580. The procedures were performed as described by the manufacture. Fluorescence-labeled samples were examined under a confocal laser scanning microscope.

For cytochrome *c* and AIF staining HeLa cells grown and infected on coverslips (shell vial cultures) were washed in PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. The slides were incubated with 1:100 dilutions of rabbit polyclonal antibodies to cytochrome *c* or AIF for 1 h at 37 °C. As secondary antibody Texas Red-conjugated goat anti-rabbit IgG (H+L) was used at a dilution of 1:50. Washings between antibody addition were performed with PBS three times. Finally, the cells were stained with Hoechst 34580. Images were taken with a confocal laser scanning microscope.

### **2.2.7 Detection of the caspase-3 /7 activity**

Cells were grown and infected on 96-well microtiter plates. The caspase-3/7 activity was measured using a Caspase-Glo<sup>®</sup> 3/7 Assay (Promega). The assay provides a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase activity, luciferase activity and cell lysis. Following caspase-3/7 cleavage, a substrate for luciferase is released, resulting in the luciferase reaction and the production of light. Luminescence was measured by a plate-reading luminometer, and the activity of caspase-3/7 is presented as the number of relative light units (RLU).

### **2.2.8 Immunoblotting**

Cells were lysed in radioimmunoassay buffer for 30 min on ice. Cell lysates were centrifuged in a microcentrifuge at 5,000 g at 4 °C for 15 min, and the supernatants were mixed with an equal volume of Laemmli sample buffer. The samples were electrophoresed

on 10-12% polyacrylamide-SDS gels. Transfer of fractionated proteins to nitrocellulose membranes was carried out with a semidry transblot system (Bio-Rad, Munich, Germany) using Bjerrum and Schafer-Nielsen transfer buffer. Blots were blocked with 4% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing 0.05% Tween 20 for 4h.

Blots were incubated with 1:1,000 dilutions of rabbit polyclonal antibodies to PARP-1 (VIC 5), HMGB-1 (ab18256), Bim (BOD), Puma/bb3 N-terminal, Bad, GAPDH (FL-335), IDO (H-110) and mouse polyclonal antibodies to cHSP60, MOMP at 4°C overnight. Alkaline phosphatase-conjugated goat anti-rabbit/mouse IgG was used as secondary antibody at a dilution of 1:1,000. Blots were incubated with the secondary antibody for 2 h at room temperature. Washings between antibodies additions were performed with TBS-Tween three times for 10 min. The bands were visualized with 5-bromo-4-chloro-3-indolylphosphate toluidine salt-*p*-nitroblue tetrazolium chloride (BCIP/NBT substrate). Molecular weights of proteins and their fragments were calculated from a plot of log molecular weight vs. distance migrated for protein standards in the gels.

### 2.2.9 Cell free degradation assay

Cells grown and infected on six-well plates were trypsinized and harvested by centrifugation at  $1.800 \times g$  for 10 min at 4°C. After being washed with ice-cold PBS, the cell pellet was resuspended in 5 volumes of ice-cold buffer A. After incubation on ice for 15 min, the cells were disrupted by agitation 15 times. Nuclear pellets were collected by centrifugation at  $1,000 \times g$  for 10 min at 4°C and further extracted in buffer B. The supernatants were centrifuged at  $20,000 \times g$  for 15 min to prepare cytosolic extracts. Cytosolic extracts prepared from mock-infected and *Chlamydia*-infected cells were used as the source of enzyme and incubated with nuclear extracts from mock-infected HeLa cells as substrate containing PARP-1 and HMGB-1. After 2 h incubation at 37°C, the mixtures were analyzed by SDS-PAGE and immunoblotting.

### 2.2.10 Column Chromatography

To separate proteolytic proteins that were induced in infected HeLa cells, cytosolic extracts were applied to a Mono Q ion exchange column (GE Healthcare Life Sciences, Freiburg, Germany). An AKTA purifier 10 instruments (GE Healthcare Life Sciences) was used to run the column. Anion exchange was performed with buffer B (0.01 M Tris, 1 M NaCl, pH 7.2) and the column was eluted with buffer A (0.01 M Tris, pH 7.2). Proteolytic activities

of the fractions against PARP-1 and HMGB-1 cleavage were measured using cell-free degradation assays as described above. Proteolytic fractions were analyzed for the presence of bands by SDS-PAGE and Coomassie Brilliant Blue staining was performed to analyze proteolytic fractions for the presence of protein bands that were absent in non-PARP-1-degrading fractions. Immunoblots of proteolytic fractions were stained with a mouse monoclonal antibody to CPAF (kindly provided by Dr. V. Forsbach-Birk, Institute of Medical Microbiology, University of Ulm, Germany). Alkaline phosphatase-conjugated goat anti-mouse IgG was used as secondary antibody at a dilution of 1: 1,000.

### **2.2.11 2-D Gel Electrophoreses**

Chromatography fractions which showed proteolytic activity (23 to 27) were collected and used for two-dimensional gel electrophoresis. The 24-cm IPG strip covering a non-linear pH range of 3-11 was rehydrated overnight in buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 0.002% bromophenol blue, 0.5% IPG buffer, 1.2% De-Streak Reagent. Isoelectric focusing was carried out using the IPGphor II with a manifold ceramic tray at 20 °C with a current of 50  $\mu$ A/ strip. The amount of 300  $\mu$ g protein was loaded and isoelectric focusing was applied by the following protocol: 3 h at 300 V, 3 h at 600 V, 4 h at 1,000 V, 3 h at 8,000 V, and 24,000 Vh at 8,000 V. Prior to the second dimension the strip was equilibrated for 15 min in 10 ml equilibration buffer containing 1% DTT and subsequently for 15 min in 10 ml equilibration buffer containing 2.5% iodoacetamide. The separation in the second dimension was performed on the Ettan DALT System with 12.5% SDS-polyacrylamide gels containing rhinohide as a gel strengthener (30 min 30 W, 4 h 100 W for six Ettan Dalt gels). Protein standard Mark 12 was used for compare the molecular weight of proteins. The gel was fixed for 2 h in a fixing solution, washed twice with ultrafiltrated water for 20 min and stained overnight in a gel staining solution. The gel was further neutralised (0.1 M Tris-base titrated to pH 6.5 with  $\sigma$ -phosphoric acid) for 5 min and washed in 25% methanol for 1 h. The stained gel was scanned and calibrated with Labscan software 5.

### **2.2.12 Mass Spectrometry**

Protein spots were excised manually. Tryptic digest of proteins and MALDI-TOF were carried out in Hans Knöll Institute, Jena, Germany. The protein spots were digested

according to the protocol of Bruker. MALDI-TOF spectra were generated with an Ultraflex I device (Bruker Daltonics, Bremen, Germany).

Peptide mass fingerprint (PMF) was submitted for identification using MASCOT interface (MASCOT 2.1.0) or ProFound. The masses were compared to peptide masses generated by a theoretical tryptic cleavage of proteins present in databases (<http://au.expasy.org>) for protein identification.

### **2.2.13 Enzyme-linked immunosorbent assay (ELISA) for HMGB-1 and PARP-1**

For enzyme immunoassays, supernatants of infected and mock-infected cultures were centrifugated at  $14,000 \times g$  for 5 min and stored at  $-80^{\circ}\text{C}$  until assayed. Levels of extracellular HMGB-1 were measured by HMGB-1 enzyme-linked immunosorbent assay according to the manufacturer's protocol.

PARP activity in cell lysates was assayed using the universal colorimetric PARP assay kit according to the manufacturer's instructions. The test principle is based on the incorporation of biotinylated Poly (ADP-ribose) onto histone proteins. Poly (ADP-ribosylation) of histone proteins was detected using streptavidin-HRP. Briefly, cell lysates from uninfected and *C. trachomatis* infected cells containing 20  $\mu\text{g}$  of protein were loaded into a 96-well plate coated with histones and biotinylated poly ADP-ribose, allowed to incubate for 1 h, treated with strep-HRP, and read at 450 nm in a spectrophotometer.

### **2.2.14 PARP-1 RNA interference and transfection**

HP validated small interfering RNA (siRNA) targeting PARP-1 (SI02662989) and nonsilencing All Stars Negative Control siRNA were purchased from Qiagen (Hilden, Germany). HeLa cells were transfected with 5 nM siRNA using HiPerfect transfection reagent (Qiagen) according to the manufacturer's protocol. At 16 h post transfection, the medium was changed to remove the transfection reagent. At 48 h post transfection, cells were further infected by *C. trachomatis* at m.o.i. 5, PARP-1 expression level and cell death induction and were assayed as described above.

### 2.2.15 Lactacystin treatment and *Chlamydia* replication assay

Lactacystin was dissolved in DMSO at a concentration of 1,1 mg/ml. To inhibit CPAF activity HeLa cells grown in 6-well plates were treated with 11 µg lactacystin after *C. trachomatis* infection (final concentration 10 µM). Control wells of mock-infected and infected cells included DMSO at an equivalent dilution. After 48 h cell lysates were prepared in radioimmunoassay buffer for SDS-PAGE. In comparison infected cells cultured in medium alone were collected in radioimmunoassay buffer and then treated with 11 µg of lactacystin for 45 min on ice. Chlamydial inclusions in HeLa cells treated with or without lactacystin were quantified after immunofluorescence staining with FITC-conjugated antibody to the *C. trachomatis* MOMP.

To assess the production of new infectious elementary bodies during the intracellular cycle, infected cells were scraped into 0.5 ml of saccharose phosphate buffer at 48 h after infection and lysed by sonication (ten pulses at 110 W; Branson Sonifier W-250). To titrate the inclusion-forming units (IFU) of the chlamydial suspensions BGM cells were infected with serial dilutions by centrifugation and incubated in serum-free Panserin medium for 48 h. Chlamydial inclusions were counted after immunofluorescence staining to determine the IFU per shell vial. The chlamydial yield was calculated from the ratio of IFU to the number of inclusions.

### 2.2.16 RNA extraction and reverse transcription (RT)-PCR analysis

Total RNA was prepared from cell monolayers using the RNeasy Mini Kit according to the manufacturer's instructions. To avoid the DNA contamination for certain RNA applications that are sensitive to very small amounts of DNA, the residual DNA were be removed by optional on column DNase digestion using the RNase-Free DNase Set. To remove the First-strand cDNA was reverse transcribed from 1 µg of RNA in a total reaction volume of 20 µL with 15 U of avian myeloblastosis virus reverse transcriptase and 0.5 µg of oligo(dT)<sub>15</sub> primer, using the Promega reverse transcription system as instructed by the manufacturer.

Each 25 µL of PCR mixture contained 0.5 µL of cDNA (corresponding to 25 ng of RNA), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.2 mM each dNTP, 1.5 mM MgCl<sub>2</sub>, 0.4 µM sense and antisense primers, and 1.25 U of *Taq* DNA polymerase (Promega). cDNA was amplified for pyruvate dehydrogenase (PDH) as reference. Thirty cycles of amplification

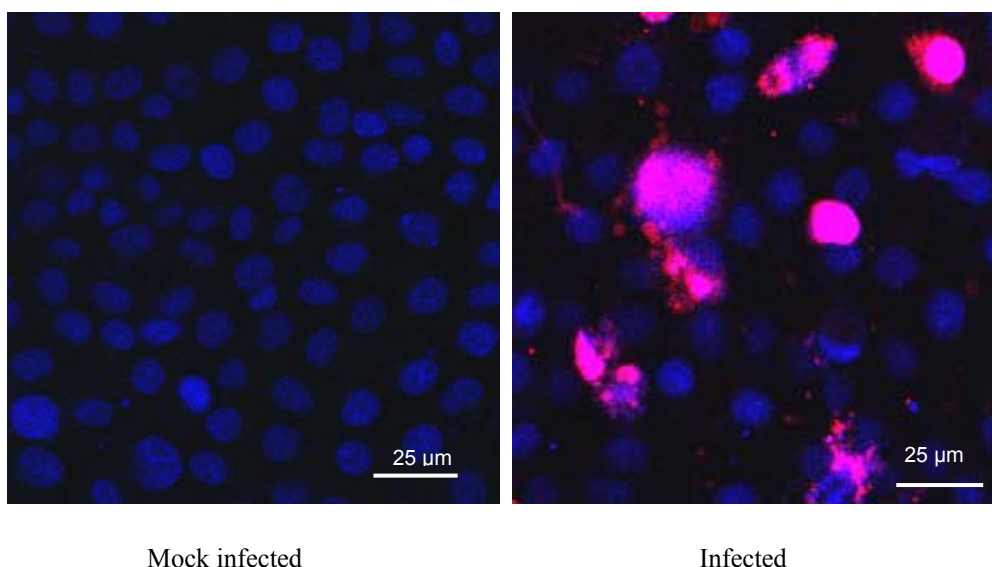
were carried out in a TRIO-Thermoblock. Reactions consisted of an initial incubation at 95 °C for 7 min and then cycling at 95 °C for 30 s, 55 °C (annealing temperature) for 30 s, and 72 °C for 1 min, with a final incubation at 72 °C. Negative controls were performed by omitting RNA from cDNA synthesis and PCR amplification. Products were electrophoresed on 1 % agarose gels containing SYBR Green I and visualized on a UV transilluminator.

### 3 Results

#### 3.1 Regulation of host cell death by *C. trachomatis* active infection

##### 3.1.1 *C. trachomatis* active infected cells induce necrotic-like cell death

As an intracellular pathogen, *Chlamydia* is known to induce severe host cell damage and subsequent inflammation. HeLa cells were infected with *C. trachomatis* serovar D at a multiplicity of infection (m.o.i.) of 5 and double-stained with Hoechst 34580 and propidium iodide (PI). Hoechst 34580 (excitation/ emission maxima  $\approx 350/ 461$  nm), is a type of blue-fluorescence dye which can bound to DNA. PI, a red-fluorescence dye (excitation/ emission maxima  $\approx 535/ 617$  nm), is only staining the cells with membrane damage. As shown in Fig. 3. 1, a numerous PI-positive cells were present in the cultures at 48 h post infection, indicating that cell death is induced following chlamydial infection.



**Fig. 3. 1** Induction of cell death in response to *C. trachomatis* infection.

HeLa cells were infected at an m.o.i. of 5 and stained with Hoechst 34580 (blue) and PI (pink) at 48 h post infection. Mock infected cells were used as control.

For flow cytometry double-labeling of the cells with annexin V and PI was used. Annexin V binds phosphatidylserine which is typically exposed on the outer leaflet of apoptotic cell membranes. PI was used for the discrimination of necrotic cells from the annexin V-

positively stained cell clusters. As the result shown in Fig. 3. 2 A, after *C. trachomatis* D infection both PI and annexin V double-positive cells was observed. The cell death induced by *Chlamydia* is significantly increased at a time dependent manner. At the earlier stage of infection (10 h p.i.), the cell death was only slightly induced compared to mock infected cells; however, at 24 h and 48 h p.i., about 28.8% and 34.7% cells underwent cell death, respectively (Fig. 3. 2 B).

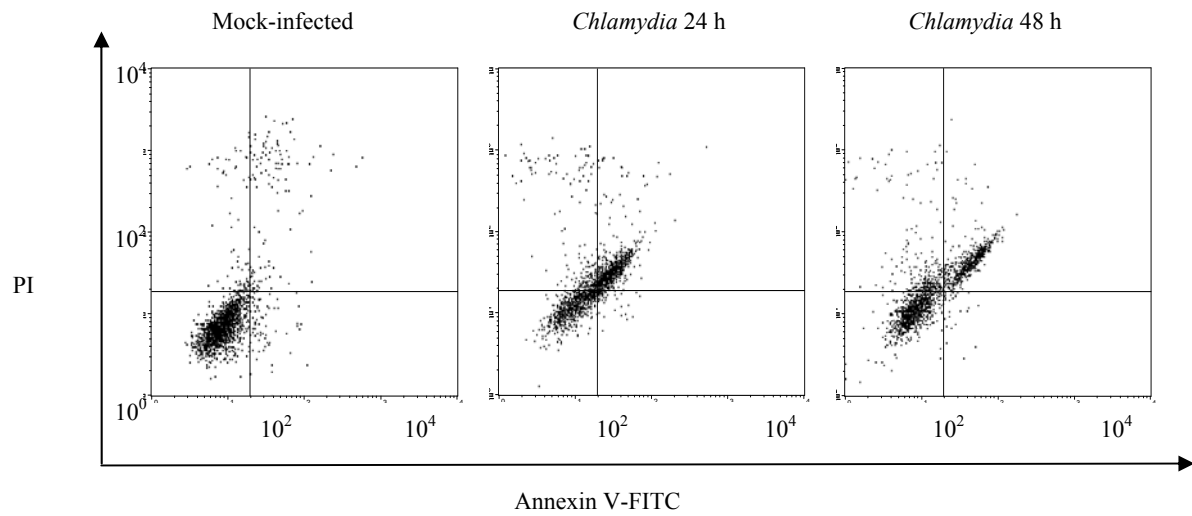
### 3.1.2 Resistance against apoptotic stimuli by *C. trachomatis* infected cells

Many studies already confirmed that *Chlamydia* showed the ability to protect infected cells against apoptotic stimuli. To evaluate whether in our cell culture model *C. trachomatis* infected cells also exhibited an extensive resistance to apoptosis despite the cell death induction, both mock infected and infected HeLa cells were treated with 1  $\mu$ M staurosporine, a protein kinase inhibitor, for 4 h. Morphology of cell nuclei was assessed by staining with nuclear dye DAPI (4'-6-Diamidino-2-phenylindole). DAPI is known to form fluorescent complexes with natural double-stranded DNA. The results in Fig. 3. 3A confirmed that under the apoptotic stimuli, uninfected cells clearly showed nuclear condensation and fragmentation, which are hallmarks for apoptosis, whereas no apoptotic nuclei were displayed in chlamydial inclusion-containing cells, proving the inhibition of apoptosis by chlamydial infection.

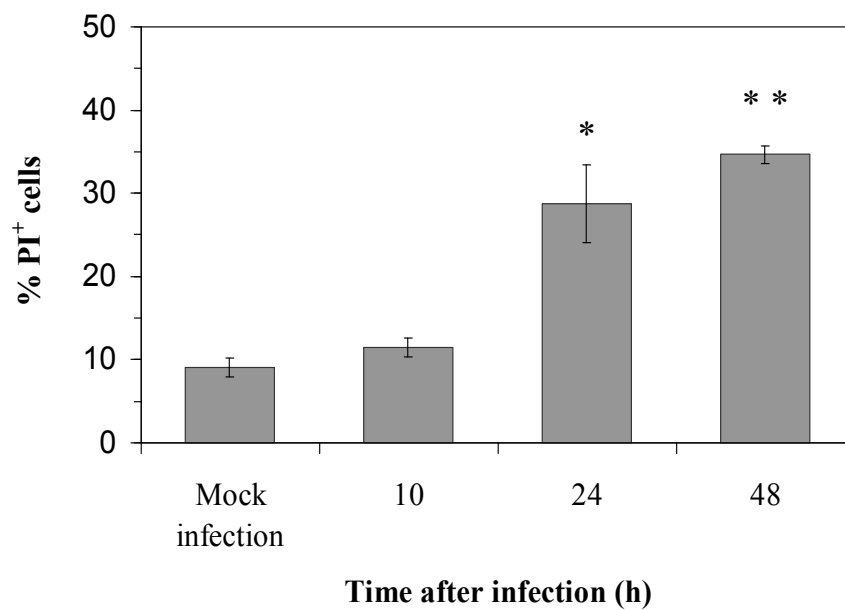
Inhibition of staurosporine-induced apoptosis was quantified by counting apoptotic nuclei under the microscope after staining with DAPI. At 24 h and 48 h post infection, the cells with apoptotic nuclei following staurosporine treatment were reduced to 5.6% and 0.9% respectively, compared to 24.1% in mock-infected cultures. But earlier stage of infection (10 h p.i.) has no clear effect on the apoptotic inhibition (Fig. 3. 3 B). These findings confirmed that the model used in this study is not contradictory to those used in studies that clearly showed the inhibition of apoptosis in chlamydial infected cells.



A.



B.



**Fig. 3. 2** Cell death during infection of HeLa cells with *C. trachomatis*, measured by flow cytometry with annexin V- PI double-labeled cells.

A. Dot blots of mock-infected and infected cells analyzed by flow cytometry with annexin V/PI double labeling.

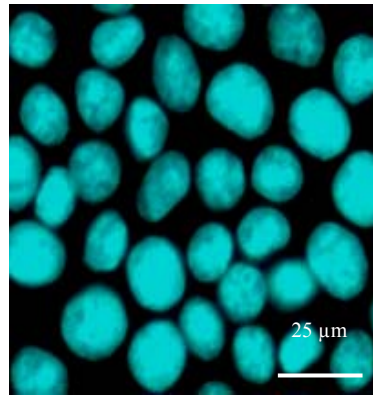
B. Time course of cell death induction by chlamydial infection. Results are given as the percentage of PI-positive cells (with standard deviations) as measured by flow cytometry.

\*,  $P \leq 0.03$ , compared with values for infected cells at 0 and 10 h ( $n = 4$ );

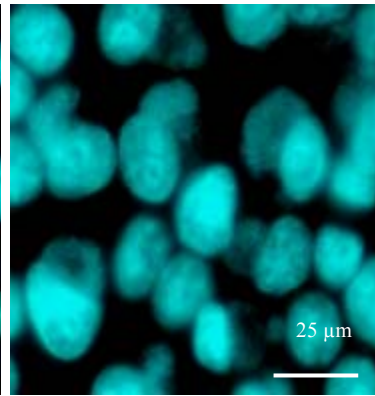
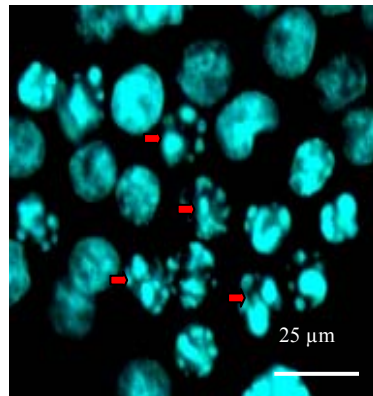
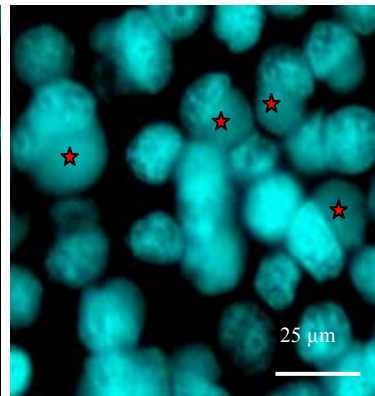
\*\*,  $P \leq 0.001$ , compared with values for infected cells at 0 and 10 h ( $n = 4$ ).

A.

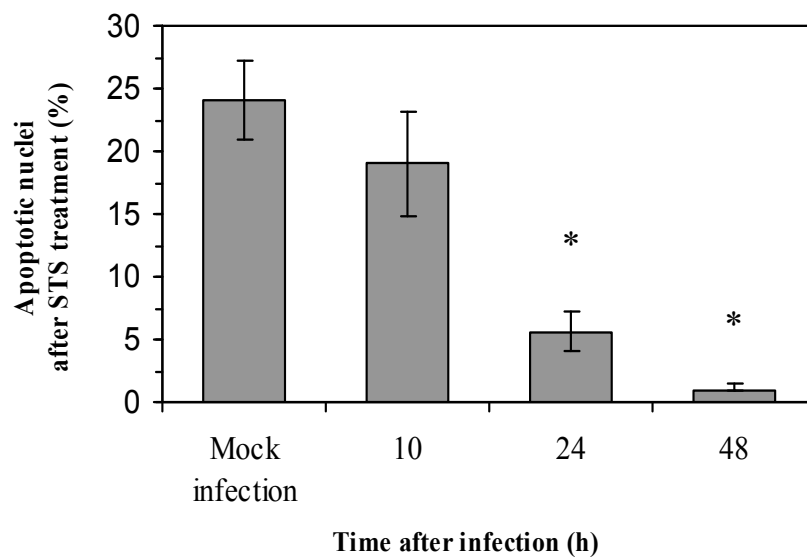
Mock infected



Infected

Mock infected  
+ STSInfected  
+STS

B.



**Fig. 3. 3** Protection of infected cells against staurosporine-mediated apoptosis

A. HeLa cells were infected at an m.o.i. of 5 and apoptosis was induced by 1 $\mu$ M staurosporine for 4 h. The morphology of nuclear was stained with DAPI. Mock infected cells used as control. (Apoptotic nuclei are indicated with arrows and chlamydial inclusions are indicated with stars.)

B. The percentage of cells with apoptotic nuclei was calculated from the determination of nuclear apoptosis after DAPI staining and counting cells in ten random microscopic fields at a magnification of 400.

\*,  $P \leq 0.01$ , compared with values for infected cells at 0 and 10 h (n = 4).

### 3.1.3 Caspase-3 is not activated during *C. trachomatis* infection

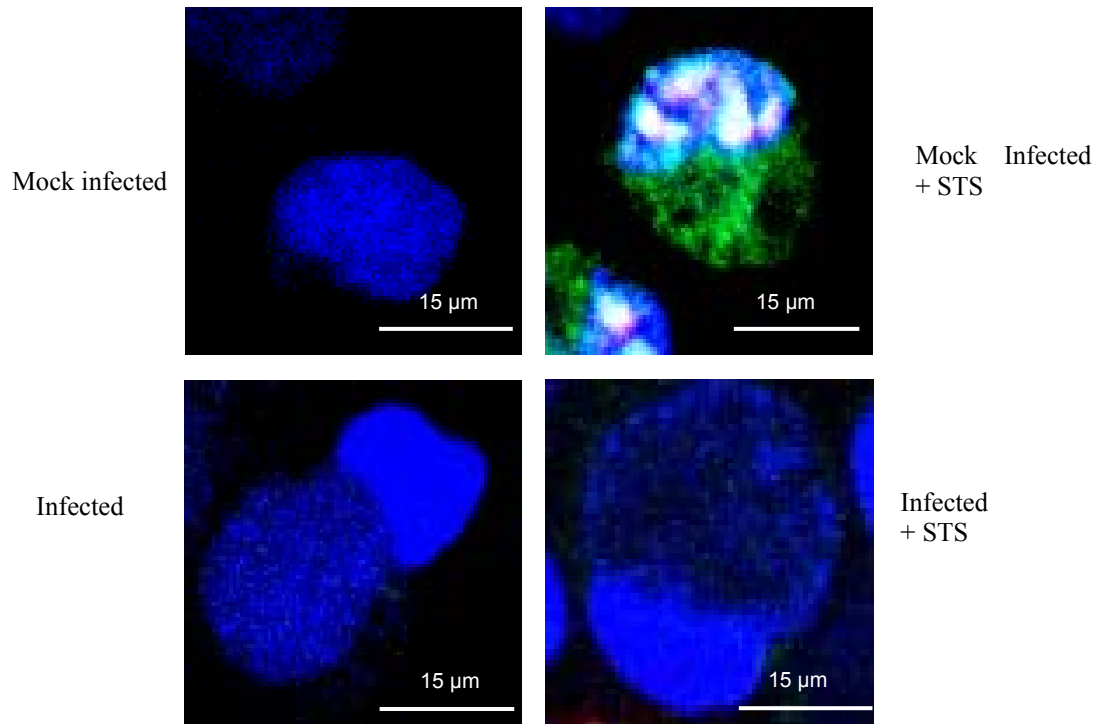
During apoptosis, the effector caspase-3 is cleaved from a non-activated 32 kDa form to an activated 19 kDa form, which cleaves and stimulates many death substrate factors like PARP-1, lamins and Fordrin. To detect if the *C. trachomatis*-induced cell death depends on caspase-3 activity, both mock infected and infected cells were stained with Hoechst 34580 (blue), annexin V (green), and a caspase-3 specific chromogenic substrate (red). Staining for the cleaved epitope that is generated during proteolytic activation of caspase-3 in apoptosis did not yield any evidence of caspase-3 activation in both cells, suggesting the *C. trachomatis* induced cell death is independent of caspase-3 activity.

As a control, apoptosis was induced using 1  $\mu$ M staurosporine for 4 h. Under apoptotic stimuli, mock-infected cells clearly showed nuclear condensation and fragments associated with positive staining for active caspase-3 and annexin V. In contrast, cells containing a chlamydial inclusion showed no nuclear fragmentation and inhibition of activated caspase-3 (Fig. 3. 4 A).

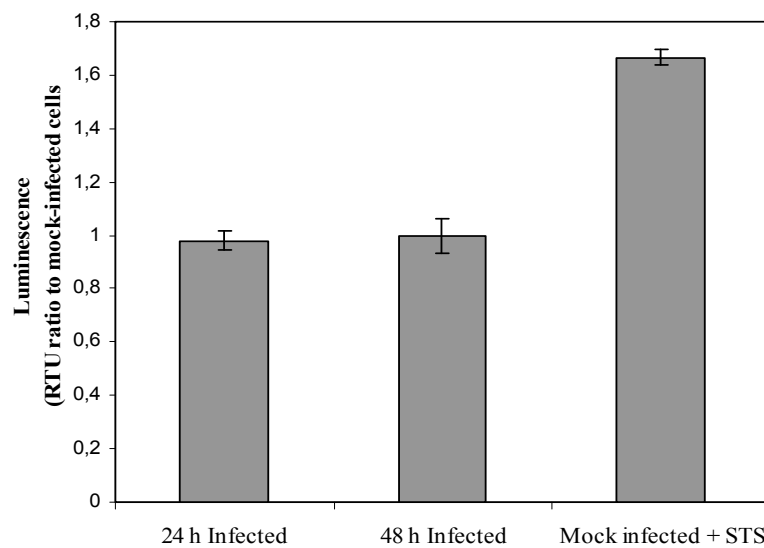
The activity of caspase-3 was quantified by using a luminescence assay. The results in Fig. 3. 4 B showed that after the treatment of staurosporine, the activity of caspase-3 in HeLa cells extraction was 1,8 fold increased, whereas the chlamydial infected cells exhibited no caspase-3 activation compared to mock infected cells.

All these results confirmed that *C. trachomatis* induced cell death is independent of caspase-3 activity and chlamydial infection could protect host cells against apoptosis by blocking the activity of this effector caspase-3.

A.



B.



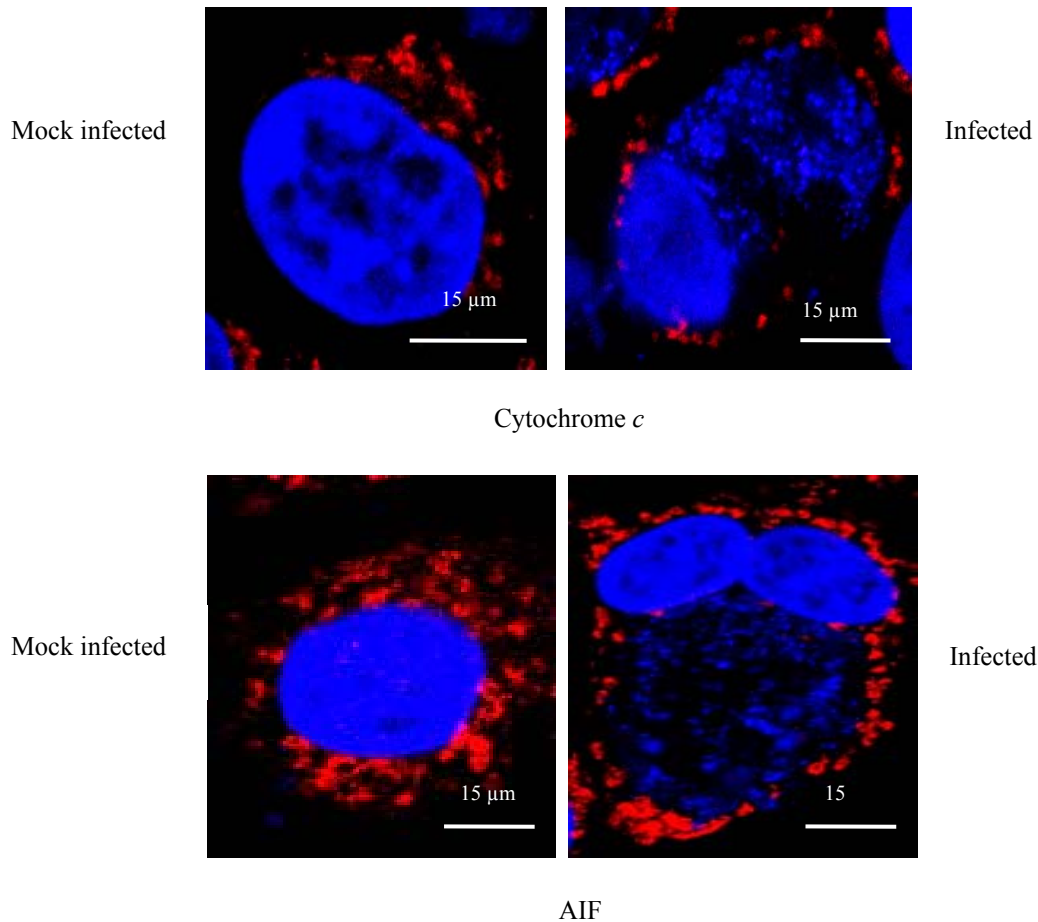
**Fig. 3. 4** Lack of detectable caspase-3 activation in HeLa cells infected with *C. trachomatis*.

A. Cells with and without infection at an m.o.i. of 5 were incubated in medium alone or treated with staurosporine for 4 h. The cell samples were stained with Hoechst 34580, annexin V, and an antibody specific for activated caspase-3. Cells were viewed under a confocal laser scanning microscope.

B. The quantitative activity of caspase-3 based on luminescence assay. The activity of caspase-3 was presented as the number of relative light units (RLU).

### 3.1.4 Mitochondrial factors are not activated during *C. trachomatis* infection

It is demonstrated that cell death during infection does not involve the activation of caspase-3. To confirm that host cell death induced by *C. trachomatis* is not mediated by the mitochondrial pathway, HeLa cells were stained with antibodies against cytochrome *c* and apoptosis-inducing factors (AIF), which are released from mitochondria to act as pro-apoptotic factors.



**Fig. 3. 5** *C. trachomatis*-induced cell death is obviously independent of the activation of apoptotic pathways.

HeLa cells were stained with Hoechst 34580 and antibodies against cytochrome *c* or AIF at 48 h after infection. Note that neither cytochrome *c* nor AIF (red) are released from mitochondria in cells containing a chlamydial inclusion.

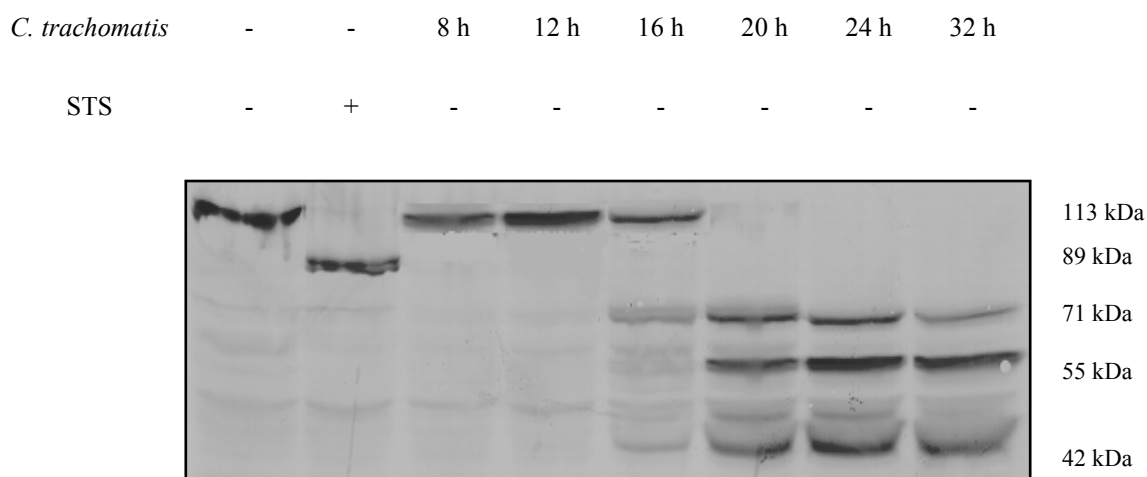
As shown in Fig. 3. 5, the cytochrome *c* in normal HeLa cells is mainly located in the mitochondria; after 48 h *C. trachomatis* infection, each infected cell was followed by a very big chlamydial inclusion, however, cytochrome *c* still displayed a clear location in mitochondria, but not a diffuse cytosol location. AIF, which would be translocated from

mitochondrion to the nucleus and directly induce nuclear fragments by caspase-independent apoptosis, was also remained in mitochondria after infection. This demonstrated that *C. trachomatis* infection failed to activate mitochondria-dependent death pathway of host cell.

### 3.1.5 PARP-1 is cleaved to a multi-form during *C. trachomatis* infection

As a nuclear DNA-binding enzyme involved in base excision repair, PARP-1 represents an important factor involved in the regulation of apoptosis and necrosis (Gobeil, S et al. 2001). It is confirmed to be cleaved into different forms when the cells are treated with different stimuli. During classical apoptosis induced by staurosporine, the full-length PARP-1 protein of 113 kDa is cleaved into two fragments of 24 and 89 kDa by activated caspase-3. While the cells undergo necrosis, it will be overactivated and cleaved to a multi-pattern (Kaufmann, S. H., 1993; Lazebnik, Y. A., 1994; Gobeil, S., 2001).

Interestingly, following *C. trachomatis* infection, PARP-1 was found to be cleaved into a ladder form with prominent bands at 71, 55, and 42 kDa, corresponding to necrotic cleavage (Fig. 3. 6). The degradation of PARP-1 started at approximately 16 h post infection, a time point at which chlamydiae are in the active phase.

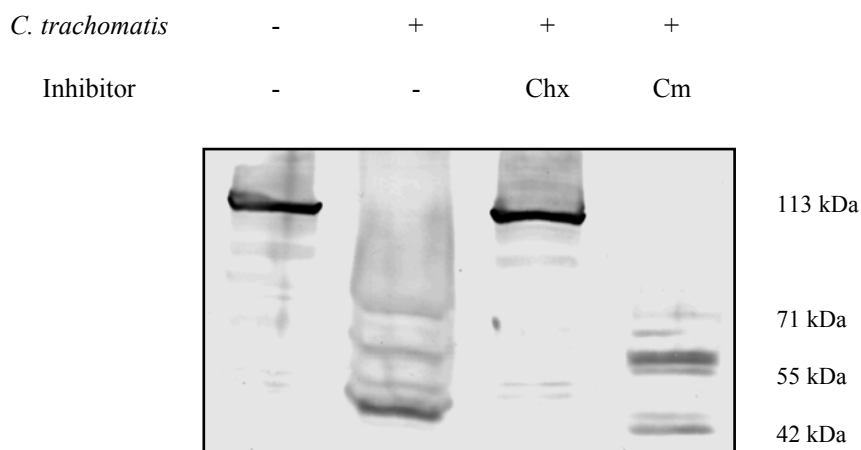


**Fig. 3. 6** Degradation of PARP-1 following infection with *C. trachomatis*

Whole cell extracts were prepared at different time points after infection and analyzed by immunoblotting. Mock infected cells and apoptosis cells induced by staurosporine were used as control.

### 3.1.6 PARP-1 cleavage depends on chlamydial but not host protein synthesis

To determine whether prokaryotic or host cell protein synthesis is required for PARP-1 cleavage, infected cells were treated with chloramphenicol and cycloheximide, respectively. Chloramphenicol is known to suppress only prokaryotic but not eukaryotic protein synthesis at the concentration used in the experiment, while cycloheximide is an inhibitor of eukaryotic translation, but has no effect on prokaryotic cells. As shown in Fig. 3. 7, cycloheximide did not prevent the degradation of PARP-1, suggesting that the new synthesis of host cell proteases is not required. In contrast, full-length PARP-1 but no cleaved fragments were detected in cells treated with chloramphenicol, indicating that a chlamydial proteolytic factor causes PARP-1 fragmentation.

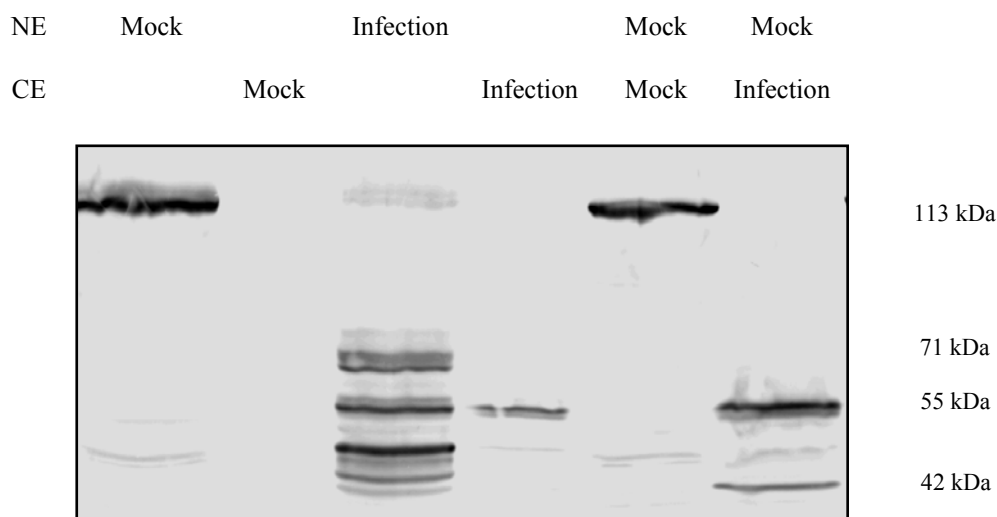


**Fig. 3. 7** Effects of cycloheximide and chloramphenicol treatment on the fragmentation of PARP-1 in infected HeLa cells (m.o.i. 5). Cm, chloramphenicol, 60 µg/ mL; Chx, cycloheximide. 10 µg/ mL.

Cell-free degradation assays were used to further characterize the PARP-1-degrading proteolytic activity induced by *C. trachomatis*. Nuclear extracts (NE) from mock-infected HeLa cells were used as the source of PARP-1 protein; cytosolic extracts (CE) from *Chlamydia*-infected cells were used as the source of proteolytic factors, and CE from mock-infected cells were used as control.

As shown in Fig. 3. 8, when NE of HeLa cells were incubated with CE from mock-infected cells the PARP-1 protein keep intact. In contrast, the incubation with CE from infected cells resulted in a total cleavage of PARP-1. These observations demonstrated that the protease factors which cleaved the nuclear protein PARP-1 existed only in chlamydial infected cells.

It should be noted that the band sizes observed in the cell-free degradation assays using CE somewhat differ from those observed in NE harvested from infected cells but this may be explained by further degradation into smaller fragments resulting from the experimental conditions.



**Fig. 3. 8** Presence of the proteolytic activity degrading PARP-1 in cytosolic and nuclear fractions of *Chlamydia*-infected but not mock-infected cells.

Cytosolic and nuclear fractions were prepared from cell samples collected at 48 h after infection and analyzed by immunoblotting. Cytosolic fractions from mock-infected and infected cells were tested in a cell-free cleavage assay using nuclear fractions from mock-infected cells as the PARP-1-containing substrate.

NE, nuclear extract; CE, cytosolic extract

### 3.1.7 Induction of PARP-1 cleavage by chlamydial proteasome-like activity factor (CPAF)

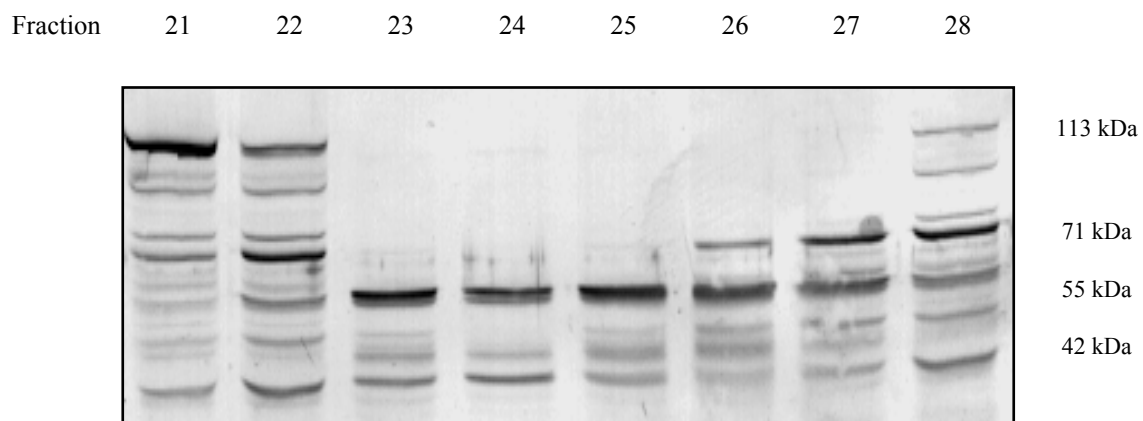
To identify the *Chlamydia*-specific proteolytic factor responsible for PARP-1 cleavage, a column chromatography approach was used to separate this proteolytic factor. A Mono Q column was used to purify fractions from cytosolic extracts of infected HeLa cells. 50 fractions eluted from Mono Q column were tested for a PARP-1-degrading activity in immunoblot assays. The nuclear extracts from uninfected HeLa cells were used as substrate. Fractions 23 to 28 showed a proteolytic activity (Fig. 3. 9 A), which correlated with the appearance of protein bands at 29 kDa in SDS-PAGE gels (Fig. 3. 9 B).

Fractions 24 to 26 were collected and separated by 2D gel electrophoresis to further separate this protein. Protein spots were cut out from a Coomassie blue-stained gel and subjected to

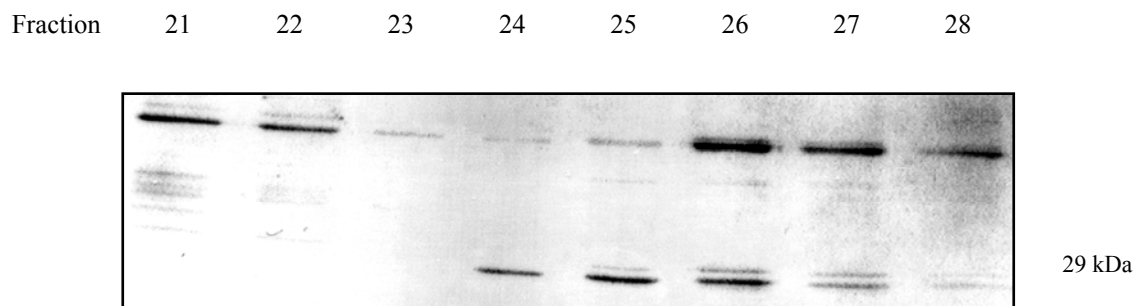


digest by trypsin for sequence identification. Using MALDI-TOF mass spectrometry analysis and comparison with the database, a spot with the estimated coordinates of 29 kDa and pI 7 matched to a protease, the NH<sub>2</sub>-terminal portion of the chlamydial protease-like activity factor (CPAF) (Fig. 3. 10 A). Immunoblots of proteolytic fractions that were stained with a CPAF antibody confirmed that the bands detected in SDS-PAGE gels represent proteins of CPAF (Fig. 3. 10 B).

A.



B.

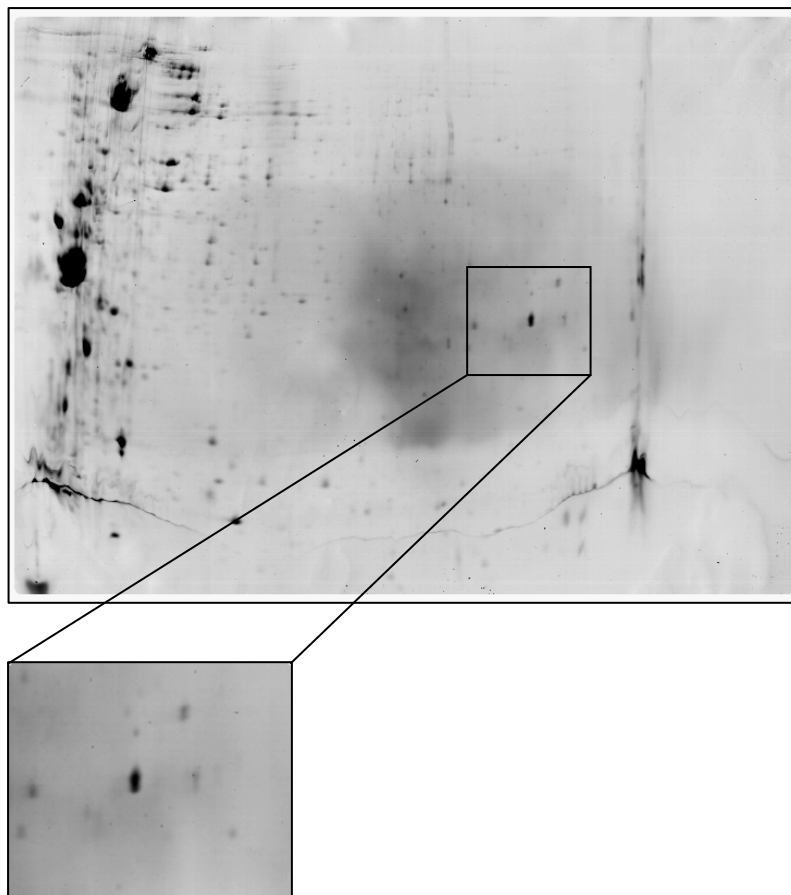


**Fig. 3. 9** Separation of proteolytic fraction from chlamydial infected cells CE by column chromatography.

A. Cytosolic lysates of cells infected at an m.o.i. of 5 were fractionated by Mono Q column chromatography. Fractions were examined for PARP-1 cleavage in a cell-free assay using nuclear extracts of uninfected cells as substrate. Cleavage of PARP-1 was detected by immunoblotting.

B. Protein profiles of proteolytic fractions were monitored by Coomassiestaining of SDS-polyacrylamide gels. Note that PARP-1-cleaving fractions showed band of 29 kDa that was absent in fractions without proteolytic activity.

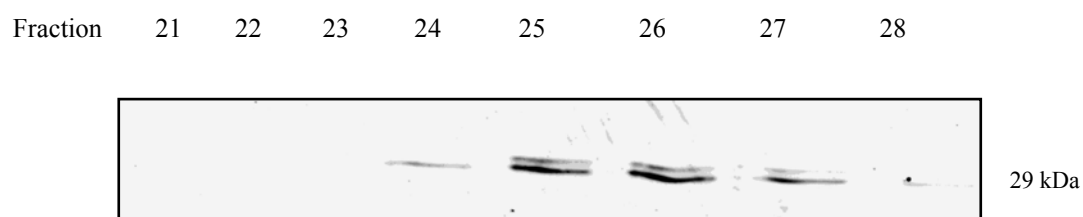
A.



CPAF: GTAAEESAALR (162-172)

YVPEGVGDLATIAPSIR (210-226)

B.



**Fig. 3. 10** Identification of CPAF as the proteolytic factor which degrades PARP-1.

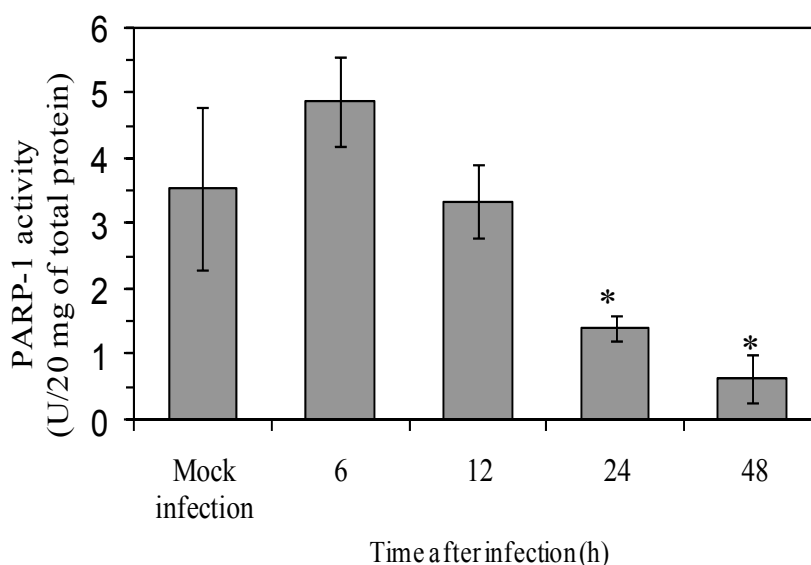
A. Proteolytic Mono Q column fractions were subjected to 2D-PAGE separation. The enlarged panel shows a 29 kDa-spot which was analyzed by MALDI-TOF and identified as CPAF.

B. Immunoblots of Mono Q column fractions were stained with a mouse monoclonal antibody to CPAF.

### 3.1.8 Cleaved PARP-1 in infected cells loses its polymerase activity

In response to apoptotic cell death, PARP-1 cleavage by caspase-3 acts as a mechanism that prevents apoptotic cells from repairing DNA. It shows a corresponding decrease in PARP-1 activity consistent with the known cleavage.

It could be supposed that the degradation of full-length PARP-1 into multiple fragments by *Chlamydia* infection also results in a loss of (ADP-ribose) polymerase activity. To detect whether the specific cleavage of PARP-1 by chlamydial infection is related to functional inhibition, HeLa cells were infected with *C. trachomatis* at m.o.i. 5, nuclear proteins were collected from mock-infected and infected HeLa cells at different time points after infection and the activity of PARP-1 was calculated by measuring the incorporation of biotinylated Poly (ADP-ribose) onto histone proteins. As illustrated in Figure 3. 11, the infection of HeLa cells with *C. trachomatis* resulted in a significant decrease of PARP-1 enzymatic activity at 24 and 48 h after infection, corresponding to the degree of PARP-1 cleavage detected in immunoblots (Fig. 3. 6).



**Fig. 3. 11** PARP-1 activity in different time course after *C. trachomatis* infection.

Each column represents the medial value from three experimetns. Total cell lysates were analyzed by a colorimetric enzyme activity assay. \*,  $P \leq 0.01$ , compared with values for infected cells at 0 and 6 h (n = 3).

### 3.1.9 Silencing of PARP-1 induces host cell death without inhibition of chlamydial developmental cycle

Next experiments should help to clarify whether the loss of PARP-1 activity in HeLa cells may promote cell death. HeLa cells were transfected with a specific siRNA to silence PARP-1 expression. Non-silencing siRNA served as a negative control. As shown by immunoblot analysis, transfection with 10 nM of PARP-1 siRNA was sufficient to inhibit PARP-1 protein expression in HeLa cells, whereas PARP-1 was presented normally following control siRNA transfection. After *C. trachomatis* infection, control siRNA-transfected cells also showed PARP-1 degradation (Fig. 3. 12 A).

As shown by flow cytometry, PARP-1 silencing in uninfected HeLa cells induced nearly 30% of PI-positive cells which was comparable to the percentage of PI-positive cells in infected cultures, while negative control siRNA exhibited similar cell death rate like uninfected cells (Fig. 3. 12 B).

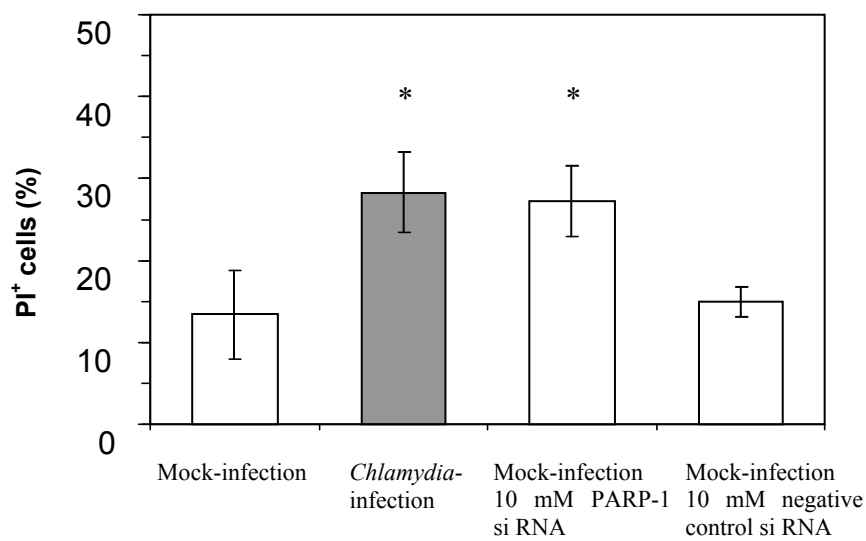
To further investigate if PARP-1 cleavage also has effects on the replication of *C. trachomatis*, HeLa cells transfected with either siRNA PARP-1 or negative control were infected with *C. trachomatis*. The chlamydial inclusions were visualised using FITC-conjugated anti-chlamydial antibody. After 48 h infection, infected cells were sonicated to harvest newly produced EBs, which can be used to infect new cells to get the titre of chlamydial yield. The graph shown in Fig. 3. 12 C gave the statistics result of 4 different experiments, which demonstrated that silencing of PARP-1 has no significant effect on the chlamydial developmental cycle compared to the negative control siRNA transfection.

A.

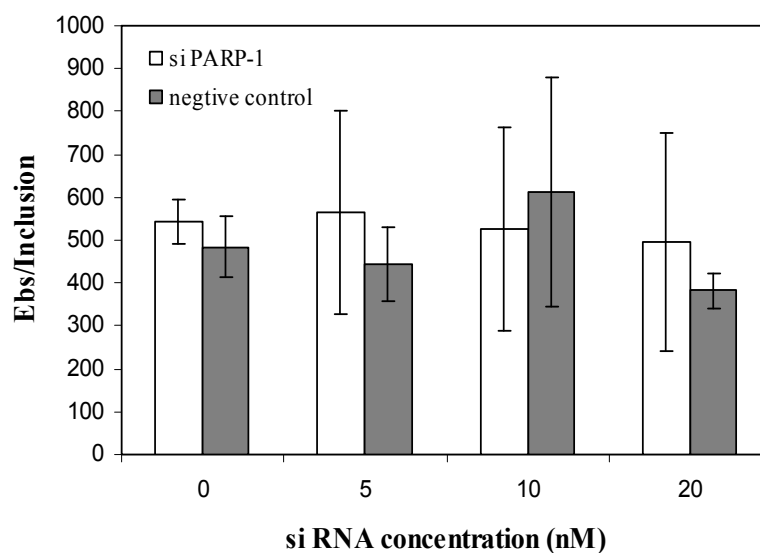
si RNA	-	-	si PARP-1		Negative control	
<i>C. trachomatis</i>	-	+	-	+	-	+



B.



C.



**Fig. 3. 12** Effect of PARP-1 silencing on the host cell death and chlamydial developmental cycle.

A. Downregulation of PARP-1 protein in HeLa cells by siRNA transfection. Cells were transfected with PARP-1-specific and nonsilencing siRNA and incubated for 48 h. PARP-1 was analyzed by immunoblotting.

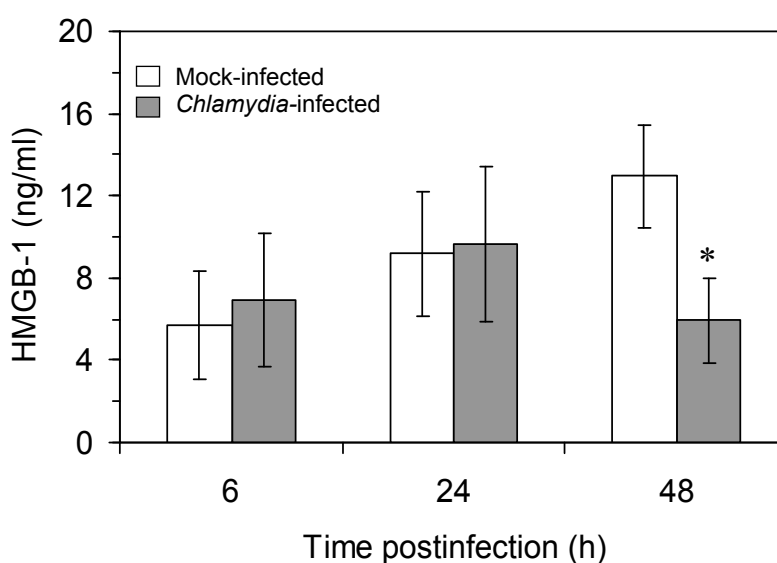
B. Cell death was measured by flow cytometry with PI labelling. *Chlamydia*-infected cells served as control. \*,  $P \leq 0.03$ , compared with values for mock-infected cells incubated in medium alone or transfected with control siRNA (n = 4).

C. PARP-1 silencing effect on the chlamydial developmental cycle. After si RNA transfection, HeLa cells were infected with *C. trachomatis* D. 48 h post infection, the EBs were collected to infect new BGM cells to measure the titre.

### 3.1.10 CPAF also contributes to HMGB-1 degradation in infected cells

High mobility group box 1 protein (HMGB-1) was identified as an important mediator of endotoxin lethality. HMGB-1 is a nuclear protein which acts as an architectural chromatin-binding factor that binds DNA and promotes protein assembly on specific DNA targets. During necrotic but not apoptotic cell death this factor can be released into the extracellular space to elicit a potent inflammatory response (Scaffidi, P., 2002).

To detect if HMGB-1 is also released during the *Chlamydia*-induced host cell death, the extracellular HMGB-1 levels were measured by enzyme-linked immunosorbent assay (ELISA). Interestingly, at 48 h after infection lower amounts of HMGB-1 were measured in supernatants of *C. trachomatis*-infected cells as compared to those of mock-infected cultures; although the uninfected cells showed the increase of HMGB-1 level corresponding to the cell proliferation (Fig. 3. 13).

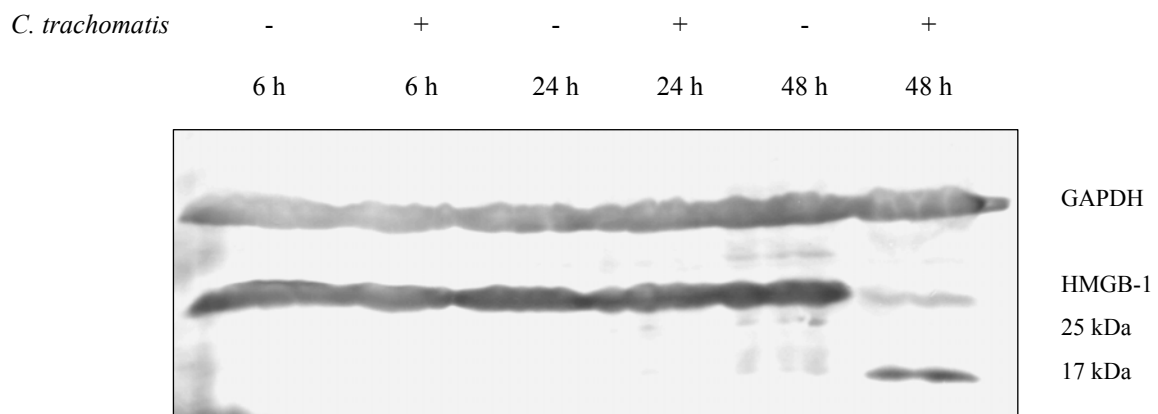


**Fig. 3. 13** Extracellular level of HMGB-1 in *C. trachomatis*-infected HeLa cell culture.

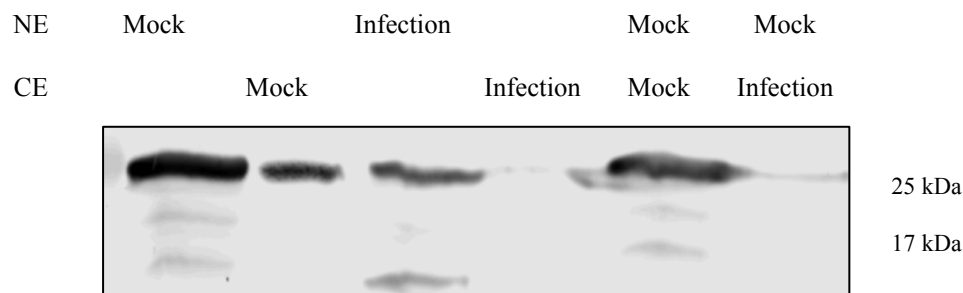
Time course of HMGB-1 released from mock-infected and infected cells. HMGB-1 levels in culture supernatants were determined by ELISA.

\* ,  $P \leq 0.05$ , compared with values for mock-infected cells at 48 h (n = 3).

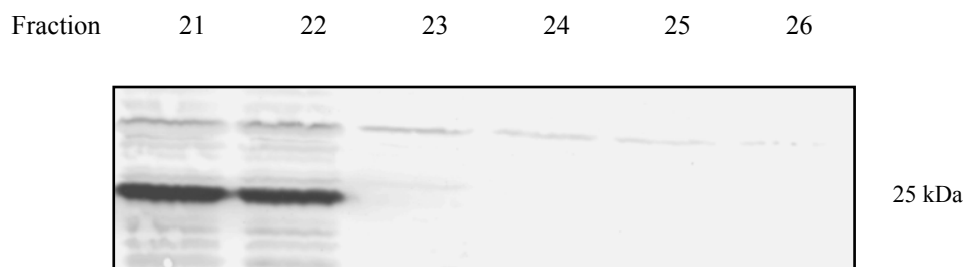
A.



B.



C.



**Fig. 3. 14** Degradation of HMGB-1 in *C. trachomatis*-infected HeLa cells.

A. HMGB-1 levels in infected cells determined by immunoblotting. GAPDH was stained as a reference band.

B. Degradation of HMGB-1 in a cell-free cleavage assay. Cytosolic and nuclear fractions were prepared from cell samples collected at 48 h after infection. Cytosolic fractions from mock-infected and infected cells were tested in a cleavage assay using nuclear fractions from mock-infected cells as the HMGB1-containing substrate. HMGB1 protein was determined by immunoblotting. NE, nuclear extract; CE, cytosolic extract..

C. Mono Q fractions of cytosolic lysates from infected cells were examined for HMGB-1 cleavage using nuclear extracts of uninfected cells as substrate. HMGB-1 was determined by immunoblotting. Note that HMGB1 disappeared in CPAF-containing fractions (Fraction 23-26, see also Fig. 3. 9).

The intracellular HMGB-1 protein level was further evaluated by Western blot. Interestingly, immunoblot analysis revealed that at 48 h after chlamydial infection, the full-length 25 kDa HMGB-1 protein was highly degraded, instead a faint smaller band of 17 kDa could be detected, which may due to a certain proteolytic degradation of HMGB-1 (Fig. 3.14 A).

Concerning that the degradation of the inflammatory factor HMGB-1 may also be induced by the chlamydial protease; the cell free degradation assay was performed to. The blot in Fig. 3. 14 B demonstrated that both cytosolic and nuclear extracts from infected cells contained lower amounts of HMGB-1 than those from mock-infected cells. Furthermore, the incubation of NE from mock-infected cells with CE from infected cells resulted in a complete loss of the HMGB-1 full-length protein.

To examine that CPAF also contributes to HMGB-1 degradation, CPAF-enriched Mono Q column fractions were tested for a proteolytic activity against HMGB-1. As shown in Fig. 3.14 C, fractions 23 to 26 caused HMGB-1 cleavage whereas fractions 21 and 22 in which no CPAF could be detected were inactive (see also Fig. 3. 9).

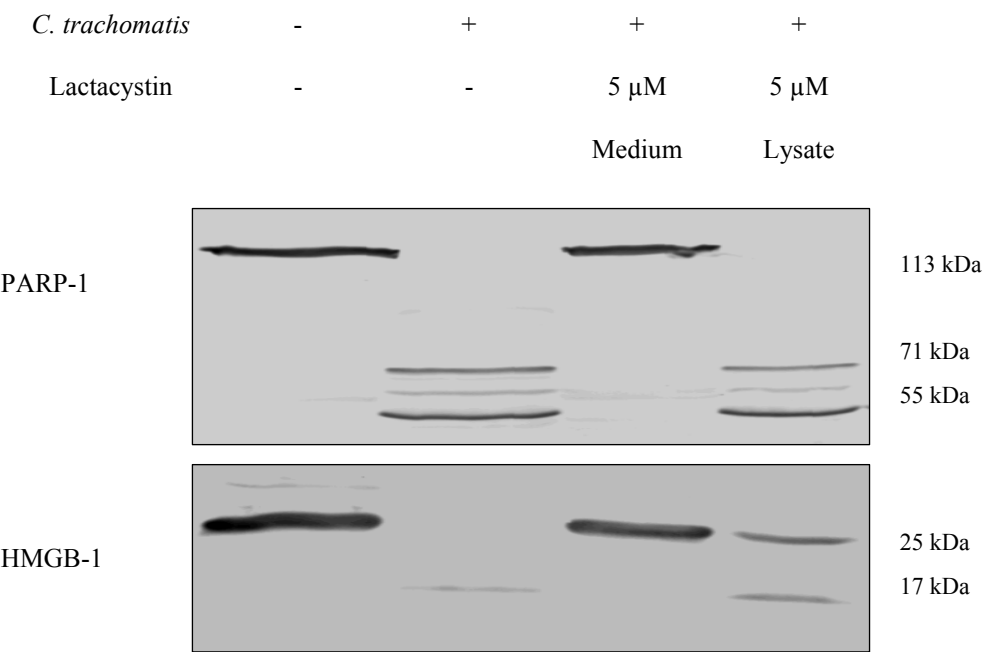
### **3.1.11 The effect of lactacystin, a CPAF inhibitor, on PARP-1/HMGB-1 cleavage and chlamydial replication**

To further confirm that CPAF functions as the proteolytic factor to degrade PARP-1 and HMGB-1, lactacystin, an inhibitor of the 26S proteasome, which has also been identified to inhibit the activity of CPAF (Zhong, G., 2001), was added to the cell culture at the final concentration 5  $\mu$ M to inhibit CPAF activity shortly after *C. trachomatis* infection. The immunoblotting shown in Figure 3. 15 also gave the result that both proteins kept intact after the inhibition of CPAF activity, which gave evidence that CPAF is necessary and sufficient to degrade the host proteins.

To exclude that PARP-1 and HMGB-1 degradation is artificially caused by CPAF during the preparation of cell lysates for immunoblotting, infected cells cultured in medium alone were collected and treated with lactacystin during incubation in lysis buffer. In this case, lactacystin had no effect on PARP-1 and HMGB-1 cleavage (Fig. 3. 15).

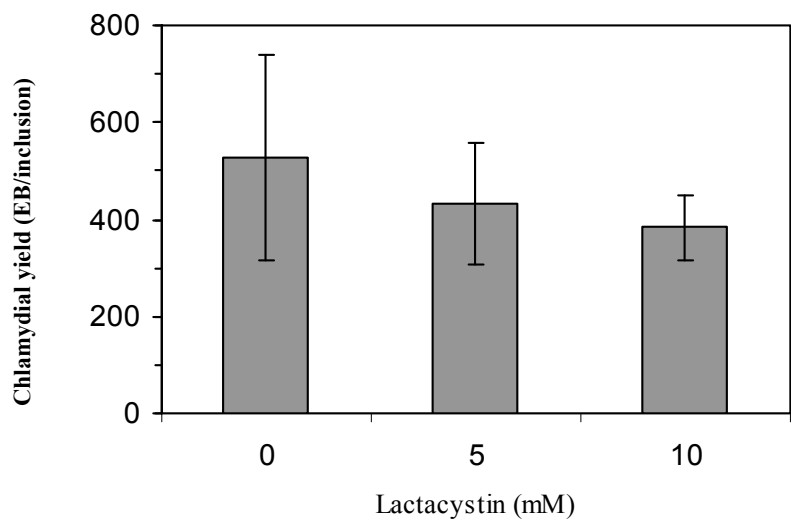
However, the addition of lactacystin on infected HeLa cells did not significantly affect the production of chlamydial elementary bodies during the replication cycle (Fig. 3. 16), suggesting that the degradation of nuclear proteins acted only as a mechanism of chlamydial interfering with host functions.





**Fig. 3. 15** Lactacystin effect on the cleavage of PARP-1 and HMGB-1.

Cells were infected with *C. trachomatis* at an MOI of 5. 11 μg of lactacystin per 35-mm-diameter well were added to the culture medium after infection (final concentration 10 μM) or to the cell harvests when lysates for SDS-PAGE were prepared. Cells were collected at 48 h after infection and analyzed for PARP-1 and HMGB-1 by immunoblotting.



**Fig. 3. 16** Replication of *C. trachomatis* in HeLa cells treated with lactacystin.

Chlamydial yield was calculated from the determination of inclusions and titration of newly produced elementary bodies. Each column was performed using three different experiments.

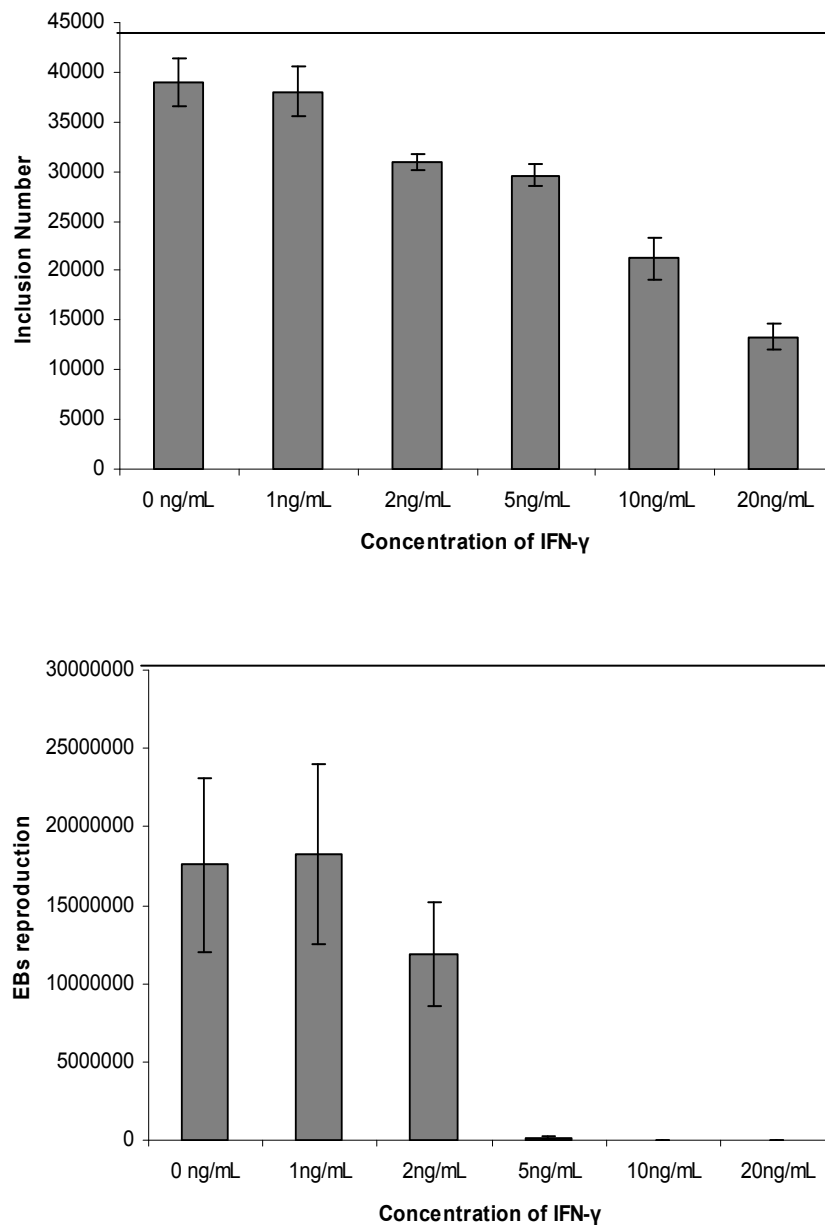
## 3.2 Regulation of host cell death by *C. trachomatis* persistent infection

### 3.2.1 IFN- $\gamma$ inhibition of *C. trachomatis* D infectivity and induction of persistence

Other studies have demonstrated that IFN-  $\gamma$  suppressed chlamydial growth and the production of infectious EBs progeny (Beatty, W. L., 1993). It is confirmed that *Chlamydia* spp. exhibited differential sensitivities to the growth inhibitory effects of IFN- $\gamma$ . *C. trachomatis* D is partly resistant to IFN- $\gamma$  because it possesses a functional tryptophan synthase and is capable of utilizing indole as a substrate for tryptophan synthesis, thus it could counteract the tryptophan depletion induced by IFN- $\gamma$  (Morrison, R. P., 2000).

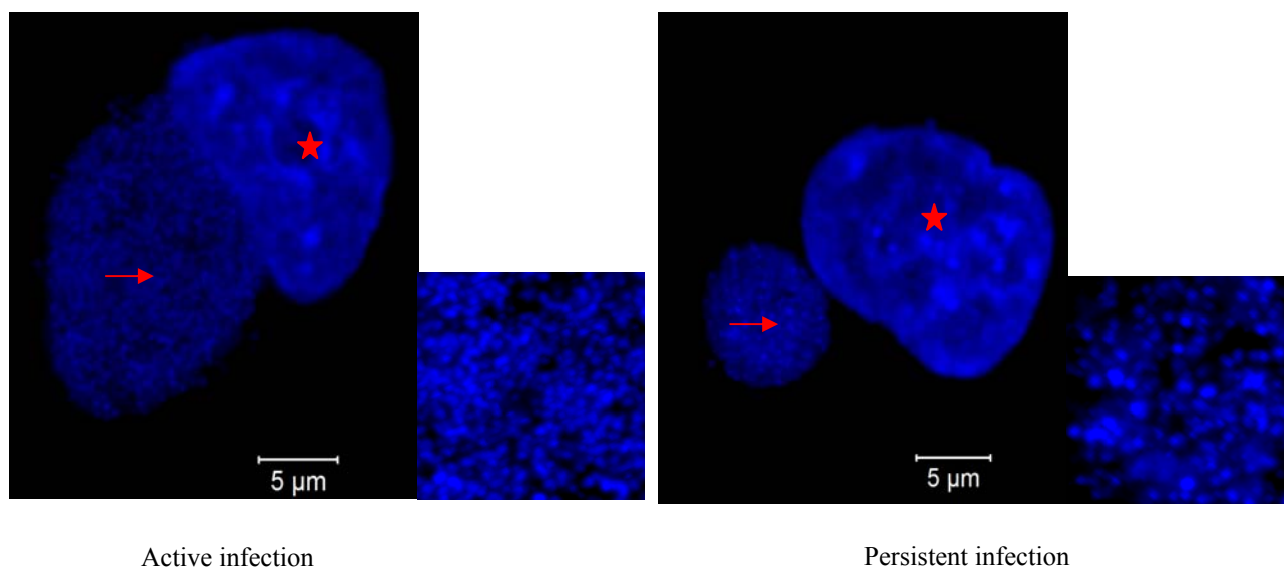
To establish the *in vitro* persistent infection model of *C. trachomatis* D, HeLa cells were pre-treated with different concentrations of IFN- $\gamma$  prior to infection to starve the tryptophan pool of the cells. After 48 h pre-treatment, cells were infected with *C. trachomatis* D at m.o.i. 5 and mock infected cells were used as control. The same concentration of IFN- $\gamma$  was added on the culture immediately after infection. The inclusions numbers were calculated after 48 h of infection and the reproduction of the EBs was measured by infecting BGM cells. Fig. 3.17 illustrates that IFN- $\gamma$  caused transition of chlamydial to the persistent state. The reproduction of EBs was drastically reduced after 48 h post infection when 5 ng/ mL IFN- $\gamma$  was added; although it has no significant effect on the inclusion numbers.

Chlamydial bodies exhibiting abnormal morphology were also observed in IFN- $\gamma$  induced persistently infected cells. Immunofluorescence images in Fig. 3. 18 revealed aberrant forms, i.e. the inclusions appeared to be smaller and RBs were larger than those seen with active, IFN- $\gamma$  untreated *C. trachomatis* infection. At higher magnification, images of aberrant inclusions revealed characteristic inhomogeneities resulting from the enlarged bodies, which distinguished them from normal inclusions.



**Fig. 3. 17** Dose response of *C. trachomatis* serovar D to IFN- $\gamma$ .

To assess the inhibitory effect of IFN- $\gamma$  on chlamydial growth, HeLa cell monolayers were pre-treated with IFN- $\gamma$  for 48 h. Cells were infected with *C. trachomatis* D at m.o.i. 5 and further IFN- $\gamma$  was added on the culture directly after infection. The inclusions numbers were calculated after 48 h of infection and the reproduction of the EBs was measured by infecting in new cells.



**Fig. 3. 18** Morphology of chlamydial inclusions during active and persistent infection.

HeLa cells monolayers infected with *C. trachomatis* D were fixed with paraformaldehyde and stained with Hoechst 34580 (blue) to show the morphology of nucleus (stars) and inclusion (arrows). 5 ng/mL IFN- $\gamma$  was used to induce persistence. To illustrate inclusion morphology, magnifications of typical chlamydial inclusions are shown in the lower right images.

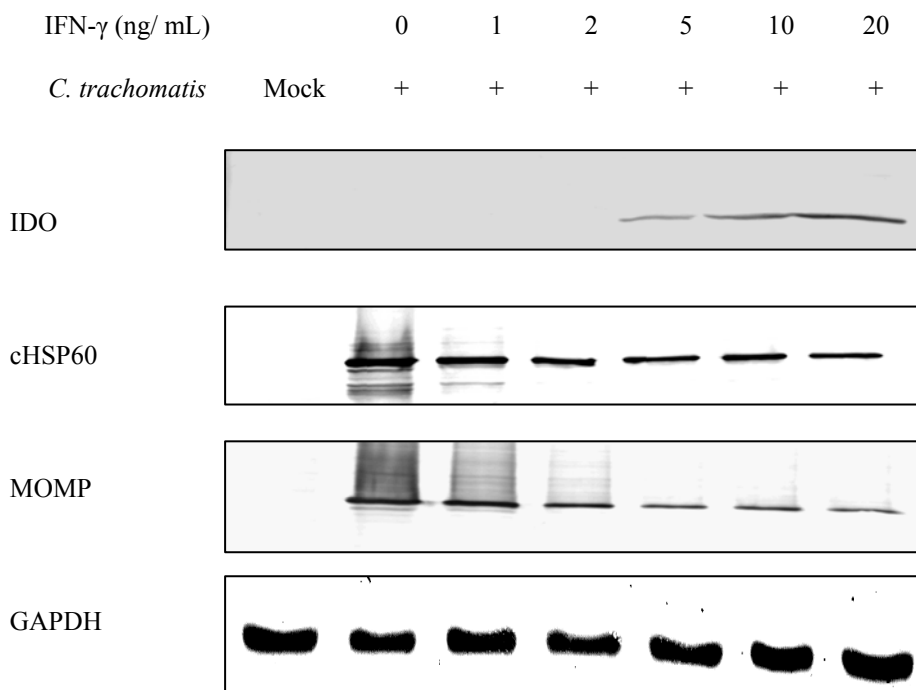
### 3.2.2 Differential protein expression during persistent infection

IFN- $\gamma$  affects human host cells *in vitro* by inducing IDO, a nonconstitutive enzyme that catalyzes the initial step in the degradation of tryptophan to N-formylkynurenine and kynurenine (Taylor, M. W., 1991, Beatty, W. L, 1994).

To confirm IDO expression induced by IFN- $\gamma$ , Western blot assays were carried out. After 48 h pre-treatment with the indicated concentration of IFN- $\gamma$ , HeLa cells were infected with *C. trachomatis* D (m.o.i. 5). The IFN- $\gamma$  was further used to induce persistence directly after infection, active non-treated infections served as controls. Total protein was isolated at 48 h post infection and expression levels of IDO related to persistence were shown in Figure 3. 19. IDO expression can be detected under the 5 ng/ mL IFN- $\gamma$  treatment, which gave another evidence that 5 ng/ mL IFN- $\gamma$  is a necessary dose for inducing *Chlamydia* persistence (see Fig. 3. 17).

In order to confirm the IFN- $\gamma$  induced persistence, the cHSP60 and MOMP proteins were investigated using western blot. Both MOMP and cHSP60 were expressed during active infection, but the MOMP protein level was highly decreased under the induction of 5 ng/ mL IFN- $\gamma$  at the same time point of 48 h. In contrast, the cHSP60 was expressed at a constant

level in persistent infection (Fig. 3. 19). This result displayed the clear decrease of MOMP-cHSP60-ratio and was consistent with the other reports (Betty, W. L., 1993), suggesting persistent state of *Chlamydia* was established after the IFN- $\gamma$  treatment.



**Fig. 3. 19** Western blot analyses of specific gene expression which is relative to IFN- $\gamma$  induced persistence of *C. trachomatis* D.

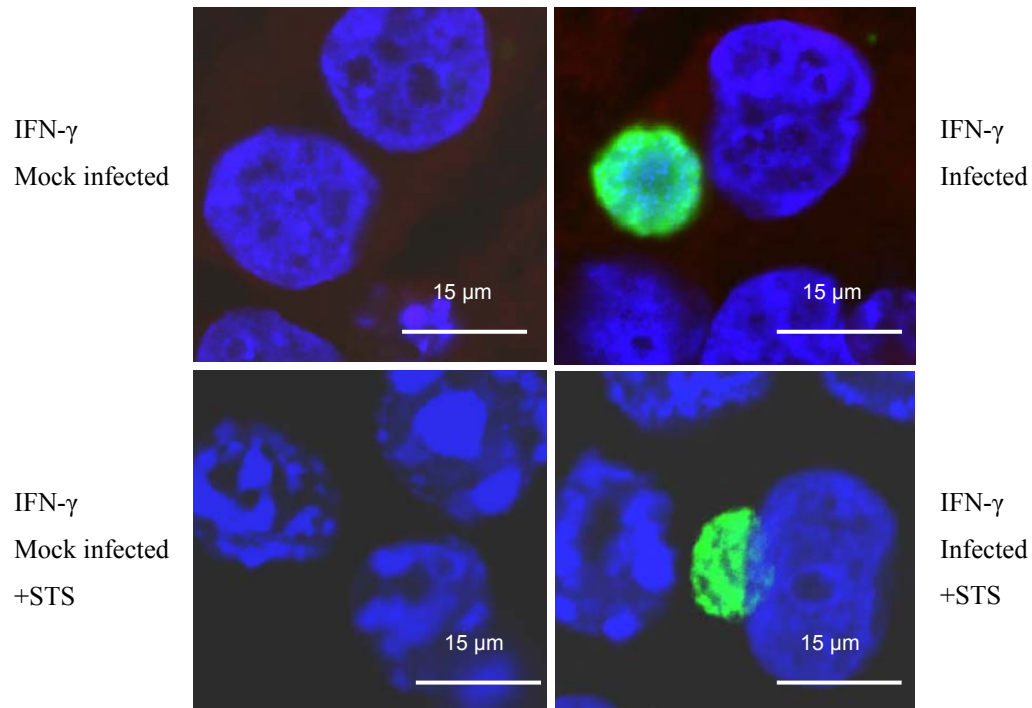
Different concentrations of IFN- $\gamma$  were used to induce persistence. Mock infected cells and active infected cells (m.o.i. 5) were used as control. Whole cell extracts were prepared after 48 h post infection and analyzed by immunoblotting.

### 3.2.3 *Chlamydia* persistence resists on apoptotic stimuli

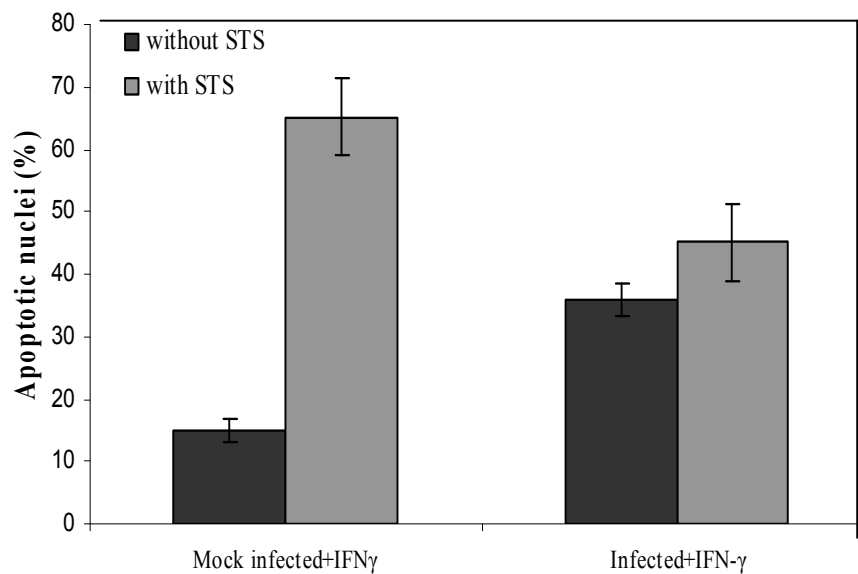
Initial studies illustrated that the active *Chlamydia* infection modulates host cell death pathways in opposite directions, both protecting infected cells against apoptotic stimuli and inducing caspase-3 independent cell death.

To detect if the apoptotic inhibition activity is also exhibited in the persistent *Chlamydia*, HeLa cells persistently infected by *C. trachomatis* D were further induced apoptosis by 1  $\mu$ M staurosporine for 4 h. To elucidate the effect of IFN- $\gamma$ , the mock infected cells were treated with the same concentration of IFN- $\gamma$  as control.

A.



B.



**Fig. 3. 20** Inhibition of apoptosis by persistent *Chlamydia* infection.

A. HeLa cells were pre-treated with 5 ng/ mL IFN- $\gamma$  for 48 h and followed by infection with *C. trachomatis* D at m.o.i. 5. After 48 h post infection, 1  $\mu$ M staurosporine was added on the cell culture for 4 h to induce apoptosis. The nuclear morphology was stained by Hoechst 34580 (blue) and the chlamydial inclusions were stained by the FITC-conjugated anti-chlamydial antibody (green).

B. The numbers counting of the apoptotic nuclei in both uninfected and persistently infected cells after the apoptotic stimuli.

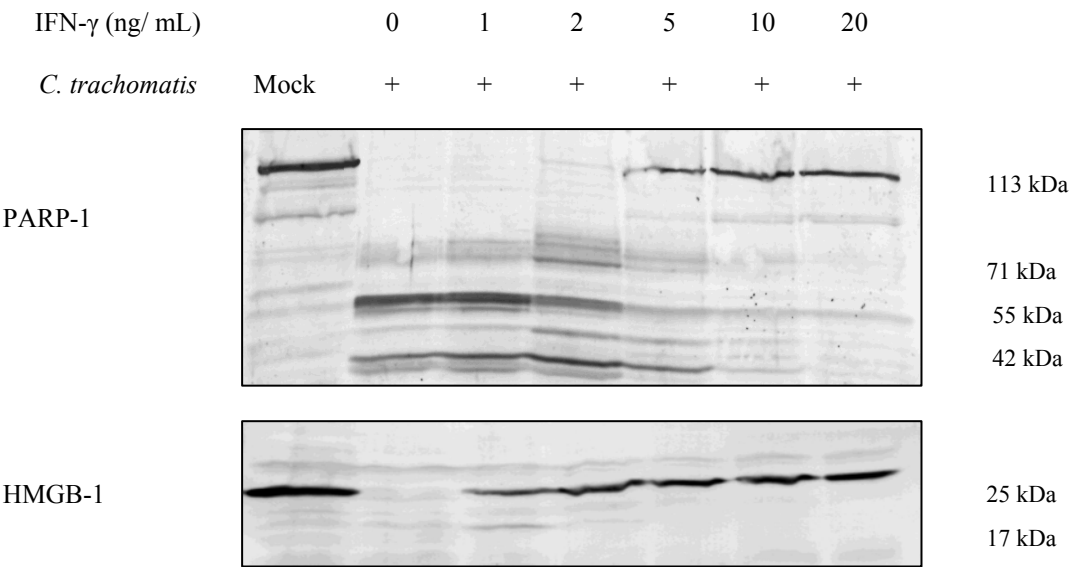
As shown in Fig 3. 20 A, after treatment with staurosporine, apoptotic nuclei existed in mock-infected cells, displaying nuclear condensation and fragmentation which are hallmarks of apoptosis. Although IFN- $\gamma$  itself induced some apoptosis in chlamydial inclusion-negative cells, whereas the cells with persistent *Chlamydia* could efficiently inhibit the apoptotic nuclear morphology.

The counting of the apoptotic nuclei gave the results that after apoptotic stimuli, the mock infected showed 50,41% more apoptotic nuclei, whereas upon the *Chlamydia* persistent infection, only 9% more cells displayed the apoptotic nuclei, suggesting the inhibition of apoptosis by the persistent *Chlamydia* infection (Fig 3. 20 B).

#### **3.2.4 Nuclear proteins keep intact during *C. trachomatis* infection**

We already showed that during *C. trachomatis* active infection, host cell nuclear proteins PARP-1 and HMGB-1 are cleaved by chlamydial protease-like activity factor (CPAF). The cleavage of nuclear proteins contributes to the chlamydial regulation host cell death pathways and prevents the inflammation factor release.

To further investigate if these factors were also related to chlamydial persistence, Western blot analysis were performed to detect these proteins in persistently infected cells. As displayed in Figure 3. 21, the nuclear proteins PARP-1 and HMGB-1 were both intact in nucleus during chlamydial persistence compared to the proteolytic cleavage pattern in active infection. The protection of these proteins against cleavage also showed an IFN- $\gamma$  dose dependent manner, by treated with 5 ng/ mL IFN- $\gamma$ , the cleavage of PARP-1 only showed a faint band in 42 kDa, whereas the HMGB-1 degradation was inhibited. This is consistent with the persistence inducing ability of IFN- $\gamma$ , suggesting that the nuclear proteins kept contact may ensure the homeostasis of the host cells during chlamydial persistent infection.



**Fig. 3. 21** Degradation of PARP-1 and HMGB-1 following persistent infection of *Chlamydia*

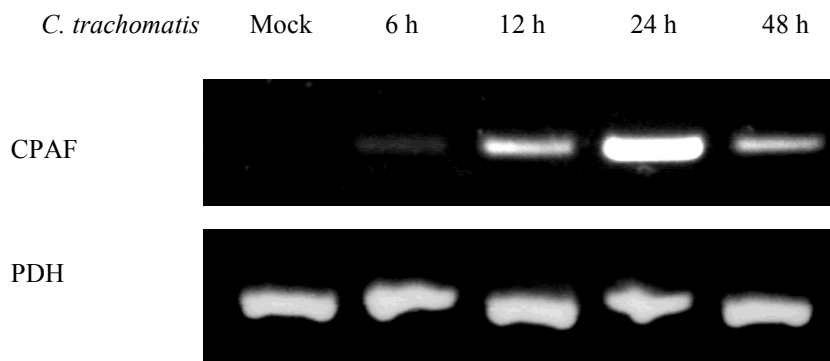
Different concentrations of IFN- $\gamma$  were added on the chlamydial infected (m.o.i. 5) cell culture. Mock infected cells and active infected cells were used as control. Whole cell extracts were prepared after 48 h post infection and analyzed by immunoblotting.

**3.2.5 Expression level of CPAF correlates with suppression of host proteins degradation during persistent infection**

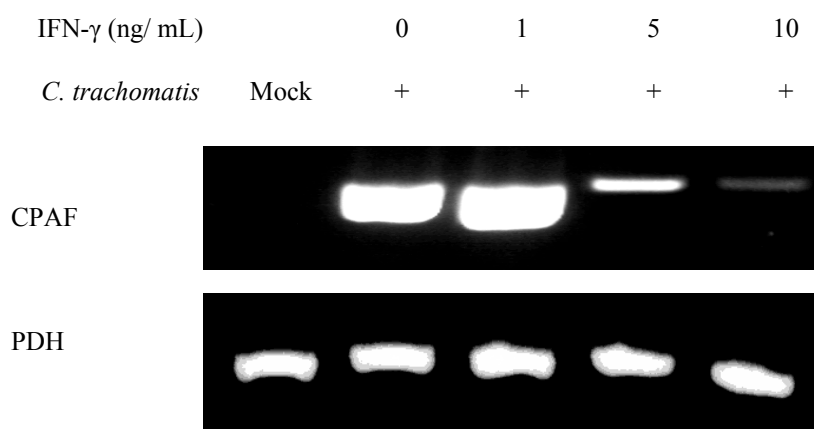
To compare the differential gene expression of CPAF in active and persistent infection, reverse transcription PCR was performed. The PCR showed that the CPAF transcription started at 12 h post infection in active infection culture, and got to maximum at 24 h post infection when the chlamydia has its most activity (Fig. 3. 22 A). However, during persistent infection, 5 ng/ mL IFN- $\gamma$  efficiently decreased but not totally inhibited the CPAF transcription (Fig. 3. 22 B), suggesting that during the persistent infection, CPAF was still expressed at a less amount compared to the mock infected control.



A.



B.



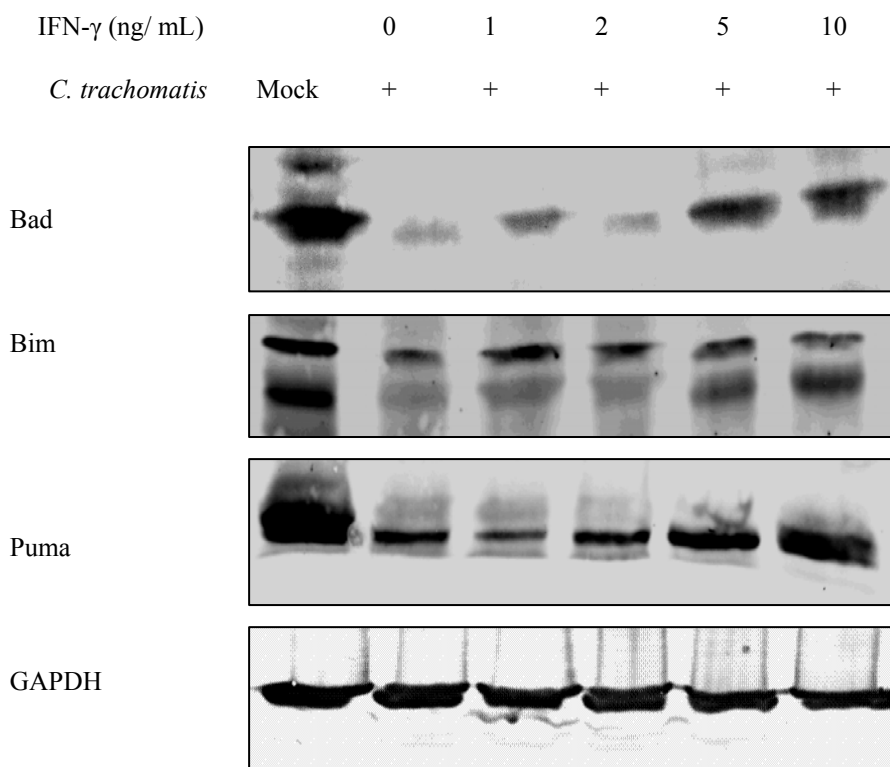
**Fig. 3. 22** CPAF transcription differences in active vs. persistent infection.

A. HeLa cells were infected with *C. trachomatis* (m.o.i. 5). Total RNA was extracted at different time points after infection, mRNA levels were determined by RT-PCR analysis. Mock infected cells were used as control.

B. Different concentrations of IFN- $\gamma$  were used to induce persistence. Total RNA was extracted after 24 h infection; mRNA levels were determined by RT-PCR analysis. Mock infected cells and active infected cells (m.o.i. 5) were used as control.

The proapoptotic BH-3 proteins (Bad, Puma, Bim) are confirmed to be substrates of CPAF during active infection (Pirbhai, M., 2006). BH-3 only proteins can transmit death signals to mitochondria by inhibiting the anti-apoptotic Bcl-2 subfamily members and/or activating the pro-apoptotic multidomain Bcl-2 family members such as Bax and Bak. Degradation of these proteins may act as one mechanism to protect the chlamydial infected cells against death stimuli like staurosporine.

Western blot assays were performed to investigate if the proteins levels of BH-3 are still corresponded to the apoptotic inhibition in persistently infected cells, as displayed in Figure 3. 23, Bim, Bad and Puma were highly decreased after 48 h active *Chlamydia* infection. During persistent infection this degradation was protected, however slight reduction of BH-3 proteins was still exhibited.

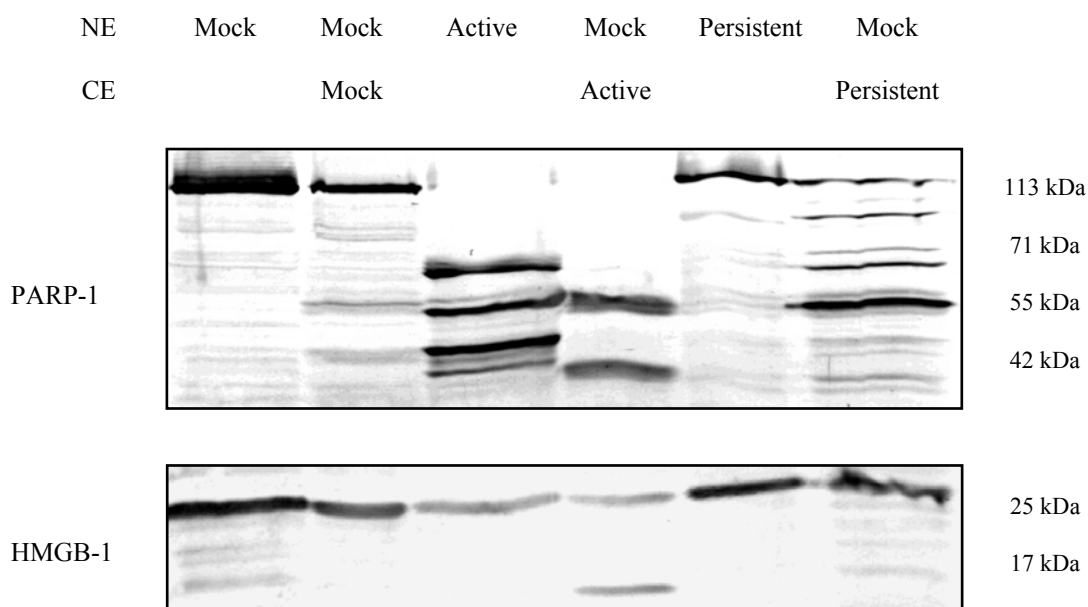


**Fig. 3. 23** Degradation of BH-3 Proteins (Bad, Bim and Puma) during persistent *Chlamydia* infection.

Different concentrations of IFN- $\gamma$  were used to induce persistence. Whole cell extracts were prepared after 48 h post infection and analyzed by immunoblotting. Mock infected cells and active infected cells (m.o.i. 5) were used as control.

To further confirm that there are still proteolytic activities existing in persistent infection, cell free degradation assays were performed as previously described. The NE from uninfected cells consisting of PARP-1 and HMGB-1 were used as the source of substrates, the CE from uninfected, actively infected and persistently infected cells were used to compare the differential activity of CPAF. As expected, the CE from actively infected cells displayed obvious proteolytic activity with the strong cleavage and degradation of both substrates. Strikingly, the CE from persistently infected cells showed the slight cleavage of

PARP-1 proteins and no degradation of HMGB-1 proteins, compared to the intact proteins in the NE of persistent cells (Fig. 3. 24), suggesting that CPAF existed only in the cytosol of persistently infected cells, but not translocated to the nuclear.



**Fig. 3. 24** Cell free degradation assay of the PARP-1 and HMGB-1 proteins.

Cytosolic and nuclear fractions were prepared from cell samples collected at 48 h after infection and analyzed by immunoblotting. Cytosolic fractions from mock-, active- and persistent-infected cells were tested in a cell free cleavage assay using nuclear fractions from mock-infected cells as the PARP-1 and HMGB-1 containing substrates. NE, nuclear extract; CE, cytosolic extract

## 4 Discussion

### 4.1 *Chlamydia* regulated host cell death pathways

The relationship between *Chlamydia* and its host is distinct in that the pathogen can exploit host cell resources throughout their intracellular bi-phasic developmental cycle. Many previous studies have confirmed that *Chlamydia* can modulate host cell death pathways to complete its own development, start a new replication cycle, and defense host cells against death stimuli.

It has been described that *Chlamydia* either induces or inhibits host-cell death during infection (Fischer, S. F., 2003; Byrne, G. I., 2004). On the one hand, to complete its special intracellular developmental cycle, *Chlamydia* effectively protect host cells against a broad spectrum of apoptotic stimuli during infection, on the other hand, induction of cell death in the late stage of developmental cycle allows propagation of chlamydia.

Initially, the apparently contradictory activities of chlamydial infection were viewed as controversial (Fields, K. A., 2002). These conflicting interpretations may be due to opposing activities (pro- and anti-apoptotic) being expressed at different levels at different times of infectious cycle. It is reported that cell death induction mostly happened in the latest stage of infection, whereas the inhibition of apoptosis existed in earlier stage. Other discrepancies would be due to the use of different *Chlamydia* strains, infectious doses or host-cell types. For instance, primary fibroblasts are more sensitive to apoptosis during chlamydia infection than HeLa cells (Miyairi, I., 2006).

Our study gives the clear evidence that *C. trachomatis* serovar D infection induces a type of cell death in epithelial HeLa cells. By flow cytometry, most of the infected cells were stained by Annexin-V and PI double positive staining, suggesting this cell death is characterized by loss of plasma membrane integrity and phosphatidylserine exposure. This death induction happened not in the beginning of the developmental cycle (10 h p.i.) but in the middle and late stage (24 and 48 h p.i.), thus it may contribute to the propagation of *Chlamydia* and act as a mechanism of disease pathogenesis by stimulating inflammatory response.

In classical apoptotic pathways, the activation of effector caspase-3 plays an important role on the death events for apoptosis. However, this apoptotic factor is not activated during

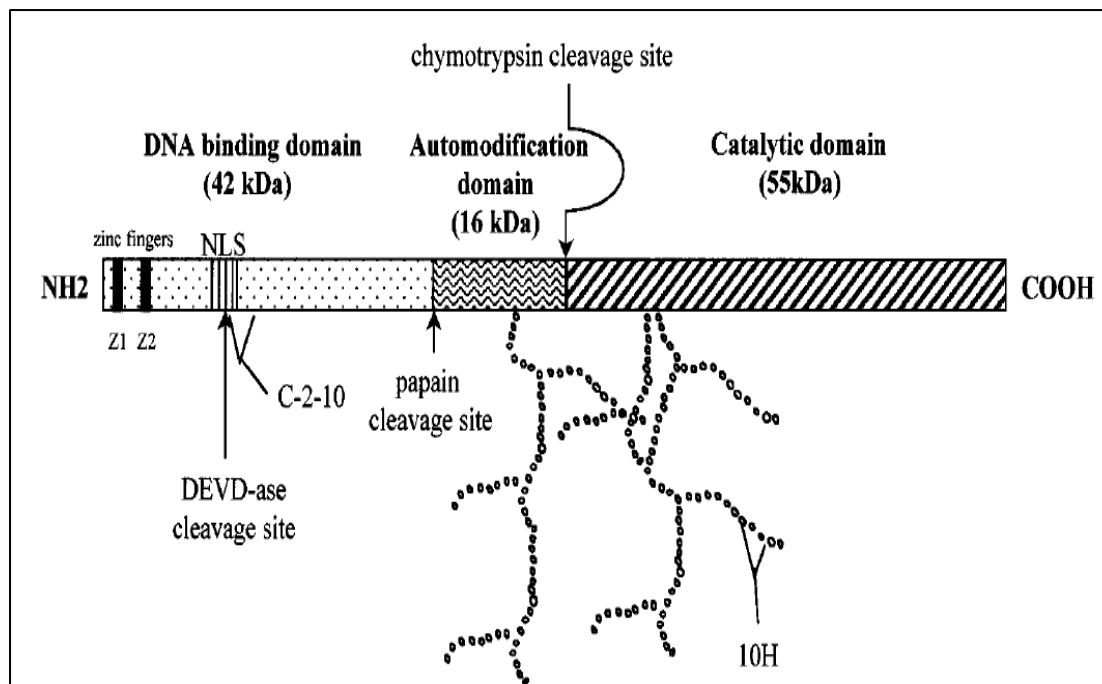
chlamydial infection. Besides, many other apoptotic factors do not correspond to the chlamydial induced cell death. For example, the pro-apoptotic factors cytochrome *c* and AIF, which are released from mitochondria and act as death signals in response to apoptosis, still remained in mitochondria after chlamydial infection. These results confirmed that cell death induced by chlamydial infection is independent of the classical apoptotic pathway.

Host response can facilitate the killing of intracellular bacteria localized in cytoplasmic vacuoles by either provoking an inflammatory response (Molloy, A., 1994; Laochumroonvorapong, P., 1996) or by delivering the intracellular pathogens to competent professional phagocytes, thus *Chlamydia* anti-apoptotic activity may be involved to suppress the host response to the initial intracellular invasion and complete its developmental cycle. As expected, when the host cells are treated by apoptotic stimuli like staurosporine, the hallmarks of apoptosis like the nuclear fragmentation and activated caspase-3 are efficiently inhibited by *Chlamydia* infection, which is also consistent with the reports from other groups (Fan, T., 1998). This result also gave another evidence that *C. trachomatis* induced cell death is independent of the caspase-3 in the classical apoptotic pathway.

## 4.2 PARP-1 cleavage during *C. trachomatis* infection

Poly (ADP-ribose) polymerase-1 (PARP-1, 113 kDa), a DNA-repair and protein-modifying enzyme, is an abundant nuclear protein that participates in the regulation of DNA repair and genomic integrity maintenance. The molecular structure of this protein comprises three main distinct regions: an N-terminal DNA-binding domain (DBD), a central auto-modification domain, and a C-terminal catalytic domain (D'Amours, D., 1999). (Fig. 4. 1)

In response to mutagenic stress signals, PARP-1 activity is stimulated more than 500-fold upon binding to DNA strand-breaks. Subsequently, it catalyzes and transfers poly (ADP-ribose) polymers onto itself and other nuclear proteins and presumably facilitate DNA repair, thereby contributing to the maintenance of genomic integrity (Lindahl, T., 1995; Shall, S., 2000).



**Fig. 4. 1** Schematic representation of the structure of poly (ADP-ribose) polymerase (PARP-1)

During apoptosis, activated caspase-3 cleaves the 113 kDa form of PARP-1 at the DEVD site to generate two fragments of 89 and 24 kDa (Kaufmann, S. H., 1993; Lazebnik, Y. A., 1994), which become a hallmark of apoptosis. This cleavage essentially inactivates the enzyme by separation of the two zinc-finger DNA-binding motifs from the automodification and catalytic domains and subsequently destroying its ability to respond to DNA strand breaks. Thus, inactivation of PARP-1 obviously prevents the depletion of  $\text{NAD}^+$  and ATP during late apoptosis (Boulares, A. H., 1998).

However, excessive DNA damage generates large branched-chained poly (ADP-ribose) polymers, which leads to the activation of a unique cell-death program. It is also apparent that an altered PARP-1 activity is a significant contributor to necrotic cell death (Gobeil, S., 2001). PARP-1 fragmentation also occurred in necrotic cell death. During necrosis, the activity of the PARP-1 is upregulated, resulting in extensive poly (ADP-ribosylation) of nuclear proteins, using  $\text{NAD}^+$  as its substrate, which in turn leads to the depletion of  $\text{NAD}^+$  and ATP energy stores inside the cells, ultimately leading to necrosis. (Zhang, J., 2000; Szabo, C., 1998; Sims, J. L., 1983). In this case, PARP-1 activity has a cytotoxic role within the cells, and the inhibition of PARP-1 with small molecules chemical inhibitor has

been shown to increase the sensitivity of cells to cytotoxic agents in a wide variety of *in vivo* (Griffin, R. J., 1996; Cosi, C., 2004).

Actually, because of the rapid activation of PARP-1, the intensity of PARP-1 activation might be a key factor that prevents the depletion of the cellular energy and regulate whether cells die or survive. Now, PARP-1 is used to distinguish apoptosis and necrosis depending on its different cleavage and activity. Strikingly, after *C. trachomatis* D infection, without the activation of caspase-3, PARP-1 is found to be cleaved into a multi-pattern form, which shows the necrotic cleavage feature (72, 55 and 42 kDa) rather than the apoptotic cleavage (89 and 24 kDa). However, the PARP-1 activity is declined after cleavage, indicating that capacity to regulate DNA repair mechanisms is impaired, and the subsequent depletion of ATP is also inhibited during the DNA damage. Thus, the loss of PARP-1 activity may silent the host reaction to DNA damage stress.

It is already known that the over activation of PARP-1 can induce necrosis and plays a role in a type of caspase-independent programmed cell death. Poly (ADP-ribose) polymer signaling mediates the translocation of AIF from the mitochondriae into the nucleus which, in turn, causes nuclear condensation (Wang, Y., 2009). From this point of view, the degradation of PARP-1 by *Chlamydia* at middle stage of the developmental cycle may contribute to strategies that prevent rapid cell death following infection. Because PARP-1 is also involved in the maintenance of cellular homeostasis and regulation of DNA repair; the silencing of PARP-1 by siRNA transfection in host cells results in the induction of cell death which is similar like that induced by chlamydial infection, suggesting that PARP-1 cleavage contributes to chlamydia induced cell death at later stage.

Concerning that PARP-1 cleavage starts at 16 h post infection when chlamydial inclusions have been developed and *Chlamydia* have high metabolic activity. PARP-1 cleavage and the loss of its activity might avoid the energy consumption of host cells and promote the replication of the pathogen.

### **4.3 CPAF causes the PARP-1 cleavage**

The time course of PARP-1 cleavage is consistent with the time course of the CPAF expression of *C. trachomatis*. After separation of this proteolytic factor from the cytosolic extraction of infected cells, analyzed by 2D-gel analysis combined with mass spectrometry, CPAF was identified as the *Chlamydia*-specific protease cleaving PARP-1.

Because the CPAF inhibitor lactacystin could prevent PARP-1 degradation when being added to the cells after infection but not when whole cell lysates were treated, PARP-1 cleavage occurs within the infected cell and was not an artificial finding resulting from CPAF-mediated effects during sample preparation.

However, treatment of infected HeLa cells with the CPAF inhibitor lactacystin had no significant effect on the progeny of chlamydial elementary bodies, which means the CPAF, as a ubiquitous enzyme secreted from chlamydial inclusion to the host cell cytosol, participating in degradation of many host proteins and acting as a very important virulence factor, seems not to be directly related to the developmental cycle of *Chlamydia*.

The *Chlamydia* genome encodes 16 proteases. For example, the CT441, a putative serine protease, could cleave p65/RelA of NF- $\kappa$ B pathway (Lad, S. P., 2007). Zhong *et al.* have firstly identified CPAF, which is synthesized as a proenzyme 70 kDa by *Chlamydia* and has to be processed into intramolecular dimers 35-kDa C-terminal and a 29-kD N-terminal subunit to acquire proteolytic activity (Zhong, G., 2001; Dong, F., 2004). This protease is secreted from the inclusion into the host cells cytosol, proteolytically activated and degrades several host proteins.

Until now, CPAF was confirmed to be responsible for the degradation of host transcription factors required for major histocompatibility complex (MHC) antigen expression such as RFX-5 and USF-1 (Zhong, G., 2001), which may allow *Chlamydia* to escape efficient detection by the immune system. CPAF further disables adaptive immune responses by degrading factors required for lipid antigen presentation (CD1d) (Kawana, K., 2007). It can also cleave cytokeratin 8 (Dong, F., 2004), which may facilitate chlamydial vacuole expansion and intra-vacuole growth. At middle stage of the infectious cycle, CPAF degrades BH-3 only domain pro-apoptotic proteins, ensuring a complete shut down of the infected cells ability to undergo apoptosis in response to intrinsic and extrinsic stimuli (Pirbhai, M., 2006; Greene, W., 2004).

Our results identified PARP-1 as a new substrate of CPAF. This is also consistent with another report from Paschen *et al.* (2008), which showed that the transfection of cells with CPAF resulted in the fragmentation of several host cell proteins including PARP-1 and induced morphological changes and cell death, suggesting that CPAF acts as a virulence factor for chlamydial pathogenicity and might be involved in causing protracted infections.



CPAF has been described to be mainly located in the cytoplasmic compartment of the host cell. At present it is unclear how CPAF is translocated into the nucleus to degrade nuclear proteins. Concerning on the activated caspase-3 translocated to nucleus during apoptosis, the same channel may be also used for translocation of CPAF. Additionally, *C. trachomatis*-infected epithelial cells typically undergo mitosis without cell division. It may be supposed that CPAF also get contact to nucleus-associated proteins during these mitotic events.

#### 4.4 HMGB-1 degradation in the late stage of infection

HMGB-1 (high mobility group box 1) chromosomal protein associates loosely with chromatin of both interphase and mitotic cells, where it regulates gene transcription. HMGB-1 has been shown to interact with and increase the apparent binding affinity of several transcription factors. In addition, HMGB-1 is also leaked rapidly into the extracellular medium when plasma membrane integrity is lost in permeabilized or necrotic cells (Müller, S., 2001; Scaffidi, P., 2002).

Jungas et al. (2004) have suggested that for both HeLa cells and primary fibroblasts, HMGB-1 was decreased following infection with *C. trachomatis*, based on the observation that HMGB-1 is lost in the cells. Interestingly, in our experiments, at a late stage of the *C. trachomatis* infection, both extracellular and intracellular HMGB-1 proteins were highly decreased compared to mock infected cells. Together with the further cell free degradation assay, it has been demonstrated that the disappearance of HMGB-1 results from certain degradation by the proteolytic factor in infected cell. The fractions from column chromatography that caused PARP-1 cleavage were also responsible for the HMGB-1 degradation, demonstrating the CPAF was related to the HMGB-1 degradation.

HMGB-1 evolves several biological functions: the intracellular form facilitates DNA binding, stabilizes nucleosome formation, and acts as a transcriptional regulator (Calogero, S., 1999). During *C. trachomatis* infection, the degradation of HMGB-1 was only happened in the latest stage (48 h post infection), but not in the earlier stage (24 h post infection), when the CPAF has already proteolytic activity to cleave PARP-1, suggesting that keeping the regulatory function of HMGB-1 in gene transcription during the infection would benefit *Chlamydia* intracellular parasite.

On the other hand, extracellular HMGB-1 released from necrotic cells has been implicated to act as a potent inflammatory mediator (Landsman, D., 1993; Giese, K., 1992; Wang, H., 1999). The activation of PARP-1 regulates the translocation of HMGB-1 from the nucleus to the cytosol, which serves to establish the ability of cells to release it upon subsequent necrosis (Ditsworth, D., 2007). Once released, HMGB-1 is able to activate several other cells involved in the immune response or inflammatory reactions, and act as a cytokine by itself. It stimulates monocytes to secrete a subset of pro-inflammatory cytokines, including TNF- $\alpha$  and IL-1 (Andersson, U., 2000). Thus, by degrading HMGB-1 in the latest stage, when large amounts of infected cells undergo cell death, CPAF may act as a virulence factor that prevents extensive inflammation, thereby contributing to immune evasion strategies of the *Chlamydia*.

#### 4.5 Chlamydial persistent infection

In recent years, it has become apparent that *Chlamydiae* can switch in cell culture and in the host organism to a metabolically and morphologically altered form so called persistence (Hogan, R. J., 2004). Chlamydial persistence is known as a state of infection during which the pathogen remains viable but non-cultivable, while the host immune system is incapable of eliminating it. It is frequently involved in chronic diseases associated with inflammatory processes, thus becomes an important issue for *Chlamydia* research.

Although the host response occurs to eradicate infection, it may also contribute to initiation of a persistent chlamydia infection. *In vivo*, the immune response against *Chlamydia* mostly depends on the inflammatory cytokine gamma interferon (IFN- $\gamma$ ). IFN- $\gamma$  suppressed *Chlamydia* mainly through the induction of IDO, subsequently leading to tryptophan starvation; which is an important amino acid *Chlamydia* obtained from host cell pool.

In this study, a persistent form was established by *C. trachomatis* D infection under the IFN- $\gamma$  stress. It is known that the different chlamydial strains exhibit differential sensitivity on IFN- $\gamma$ . *C. trachomatis* D is resistant to IFN- $\gamma$  due to its own tryptophan synthase and utilization of indole as a substrate for tryptophan synthesis. However, pre-treatment host cells with 5 ng/ mL IFN- $\gamma$  resulted in the induction of IDO, which efficiently eliminates this resistance and induce the persistence.

Morphologically, the persistent *Chlamydia* is characterized by reduced inclusion size and enlarged, pleomorphic RBs which are inhibited in binary fission and their differentiation to EBs. On the molecular level, compared with active infection, persistent bacteria exhibit altered protein expression, metabolism activity and often resistance to antibiotic therapy. For instance, immunoblot results showed that the MOMP-to-cHsp60 ratio was significantly reduced, which served as a marker of persistence. The reduced levels of MOMP, as an immunoprotective antigen, could enable chlamydia to avoid the development of protective immunity. In contrast, the steady level of cHSP60 would promote immuno-pathology through delayed-type hypersensitivity (Betty, W. L., 1993).

#### 4.6 Persistent infection resists on apoptotic stimuli

The active *Chlamydia* infection has already been demonstrated to both induce cell death and inhibit apoptotic stimuli to ensure the complete of developmental cycle and start new infection. It is known that persistent chlamydia can persist for as long as 5 years in the genital tract *in vivo* (Dean, D., 2000).

Thus the protection of the persistently infected cells against death stimuli and the host immune system may benefit *Chlamydia* to establish long-term infection. The cHsp60, an immunopathological antigen to elicit the hypersensitivity reactions of hosts, is maintained during persistence also suggesting an apoptotic inhibition mechanism may exist in persistently infected cells to ensure indefinite survival of the bacterial while expressing their most inflammatory antigen.

Herein, we confirmed that although IFN- $\gamma$  itself induced the apoptosis of eukaryotic cells in the absence of infection, persistent *C. trachomatis* infection under IFN- $\gamma$  stress still exhibited the inhibition of apoptosis induced by staurosporine. Anti-apoptotic activity is prolonged during chlamydial persistence, which strengthens the hypothesis that chlamydial metabolism maintains host cell integrity and contributes to intracellular survival.

The ability to establish a persistent infection is an important step in the pathogenesis of scarring disease seen in both trachoma and chlamydial sexually transmitted diseases. Thus, resistance to cell death by *Chlamydia* persistent infection may establish a continuous antigenic pool that stimulates ongoing inflammation and subsequent fibrosis.

#### 4.7 Persistence regulates death pathways by different CPAF expression profile

It has long been known that host factors are important in disease progression; we compared the different protein profiles during the active and persistent infection.

During active *Chlamydia* infection, CPAF cleaves nuclear proteins PARP-1 to a multi-pattern form and induces a caspase-3 independent cell death. It also degrades the inflammation factor HMGB-1 in the latest stage of developmental cycle to avoid the host immune system. However, during persistence, both proteins were detected to be intact in nucleus, giving the explanation that persistent *Chlamydia* might ensure the integrity of host cells for a long-term survival without stimulating the inflammation factors.

Cell free degradation demonstrated that the cytosolic extraction from persistently infected cells slightly degrades the nuclear proteins PARP-1 and HMGB-1. It has already been reported that little or no differential expression of CPAF between persistent and active infections (Hogan, R. J., 2004; Heuer, D., 2003). Heuer (2003) further indicated that CPAF translocation was separated from its production. The translocation mechanism was fully activated during active infection; however, during persistent infection induced by IFN- $\gamma$ , it is fully or partially inhibited.

In this study, the expression of CPAF was also compared between active and persistent infection by RT-PCR. During active infection, CPAF transcription began at 12 h post infection and arrived to the maximum in 24 h post infection, when *Chlamydia* had the most activity. However, during the IFN- $\gamma$  induced persistence, the expression of CPAF was efficiently decreased but not totally blocked; suggesting a small amount of CPAF exists in the infected cells to play a certain role in interaction with host cell signal pathway.

CPAF is also known to interfere with the apoptotic pathway by degrading the pro-apoptotic BH-3 only proteins during active infection (Pirbhai, M., 2006). BH-3 proteins locate in the cytosol of host cells and transmit death signal to mitochondria by inhibiting the anti-apoptotic Bcl-2 subfamily members or activating the pro-apoptotic Bcl-2 family members such as Bax and Bak (Willis, S. N., 2005; Bouillet, P., 2002). Our results illustrated that in persistently infected cells, BH-3 proteins were still partially degraded compare to the mock infected cells, but increased compared to actively infected cells. This

also confirms that during persistent infection of *Chlamydia*, the CPAF is slightly expressed, but the translocation of CPAF to the host nucleus is inhibited.

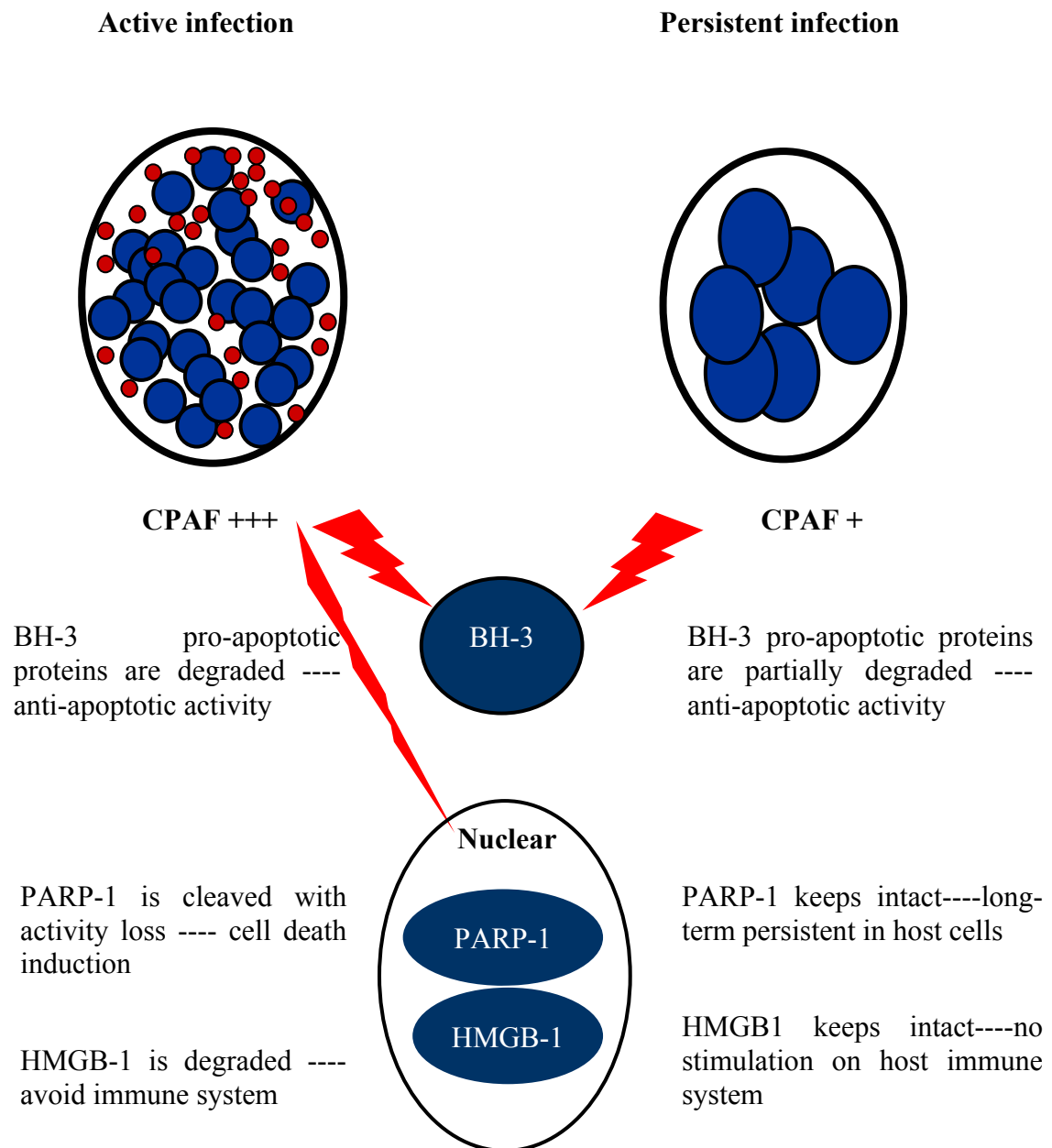
The different CPAF expression profiles in active and persistent infection may give us some explanations of how persistent *Chlamydia* can protect the host cells against some apoptotic stimuli, spontaneously keep the host cells integrity for long-term persistence.

## 5 Conclusion

*C. trachomatis* exhibits the activities of both inducing host cell death and protecting the infected cells against apoptotic stimuli. In this study, the *Chlamydia*-induced cell death was investigated. Upon infection with *C. trachomatis* D the host cell displays the characteristic of a non-apoptotic caspase-independent cell death.

*Chlamydia*-secreted protease CPAF interferes with many host functions by degrading a series of host proteins. Herein, two nuclear proteins, PARP-1 and HMGB-1, are identified as new substrates of CPAF. Upon a replicative chlamydial infection, PARP-1, a DNA repair enzyme, is cleaved by CPAF into multi-fragment during the middle and late stages of infection. This PARP-1 cleavage is related to the loss of its DNA repair activity, which is involved in the host cell death induction by *Chlamydia*. In the late stage of infection, CPAF also degrades the HMGB-1 to prevent the release of this inflammatory factor to the extracellular matrix (Fig. 5. 1 left).

Under IFN- $\gamma$  stress, *C. trachomatis* undergoes a persistent infection form, which shows low cultivability and long-term relationship with the host cells. During persistent chlamydial infection, CPAF expression is decreased. It ceases to translocate to host nucleus, thus the nuclear proteins (PARP-1 and HMGB-1) keep intact, which may ensure the long-term homeostasis of host cells without stimulating host immune system. However, the pro-apoptotic proteins BH-3 are partially decreased to protect the persistent cells against the remained death stress *in vivo*. The balance between the cell death induction and protection against death stimuli may ensure the persistent *Chlamydia* long-term intercellular survival in host cells Fig. 5. 1 right).



**Fig. 5. 1** *Chlamydia* interfere with host cells death pathways during different condition

## 6 References

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# Curriculum Vitae

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## Statement

I am familiar with the Promotionsordnung of the Faculty of Biology and Pharmacy of the University of Jena. All parts of the Dissertation were produced by myself. I hereby declare that this thesis does not contrain any material previously written or published by any other person, except where due acknowledgement or reference is made in the text. I also declare that I did not obtain the assistance of a dissertation counselling agent and that I did not provide any direct or indirect financial remuneration to any third party in connection with the content of my dissertation.

Jena, 27, August, 2009

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